TUMOR CELL RESPONSE TO DRUG INDUCED APOPTOSIS IS A FUNCTION OF INTRA-CELLULAR REDOX STATUS

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Dedicated to Amna and Aalishba

"For their love and patience during these arduous years……."

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SYNOPSIS

The term **ROS** (Reactive Oxygen Species) is used to describe mainly two products superoxide anion ($\mathbf{O_2^{\cdot-}}$)and hydrogen peroxide ($\mathbf{H_2O_2}$) that are generated within the cell and have profound effects on cell survival or cell death . We believe that a tight balance between the ratio of these two reactive oxygen species maintains cell viability. Although cancer cells have diverse features certain characteristics are commonly shared among them. We hypothesize that slightly elevated levels of superoxide anion ($\mathbf{O_2^{\cdot-}}$) levels in the cells not only promotes cell growth but in addition makes them resistant to anti-cancer drug treatment. However, if this balance is tilted in favor such that cells now produce H_2O_2 this leads to intra-cellular acidification and facilitation of apoptotic signaling.

Initially, using the photo-oxidation model of MC540 (Pervaiz, 2001) I set out to study the effect of vitamin C on the photo-oxidation of MC540 in an effort to provide an explanation for the increased sensitivity of tumor cells to PDT in the presence of vitamin C. Here the thesis discusses that the presence of vitamin C during photo-oxidation of MC540 significantly increases the apoptosis inducing potential of MC540 in tumor cells. These results indicate that the increase in tumor cells responsiveness to PDT could in part be explained by an increase in photo-oxidation of chromophore thereby yielding biologically active mixture p(MC540+VitC) that directly activates apoptotic machinery in tumor cells by stimulating intra-cellular production of H_2O_2. Next I investigated the involvement of Bax in H_2O_2-mediated apoptosis. Using human leukemia cells, I have demonstrated that exogenous H_2O_2 or H_2O_2 producing drug merodantoin (C1) induces classical hallmarks of apoptosis, by translocating Bax to the mitochondria which could be

inhibited by pre-incubation with H_2O_2 scavenger catalase. The cells have undergone a"decisive tilt" in favor of apoptosis. To substantiate the "redox hypothesis" on the fact that cells can be primed by increase production of O_2^- to become resistant to drug induced apoptosis I used another compound resveratrol (RSV). Here I provide evidence that exposure of human leukemia cells to low concentrations of RSV (4-8μM) inhibits apoptosis induced by incubation with H_2O_2, or upon exposure to anti-cancer drugs merocil (C2), vincristine and daunorubicin. At low concentrations, RSV elicits pro-oxidant properties as evidenced by an increase in intracellular O_2^- concentration. The pro-oxidant effect of RSV is further supported by our observations that the drop in intracellular O_2^- and cytosolic acidification induced by H_2O_2 or anti-cancer drugs are blocked upon pre-incubation with RSV.

Taken together I have made an effort to deduce that generation of H_2O_2 is a critical event in mitochondrial apoptosis using novel molecules like C1 or C2 derived from photo-oxidation of MC540. In addition, H_2O_2 engages pro-apoptotic proteins like Bax that will render the cells susceptible for efficient apoptosis. However, it is worth pointing out that using certain compounds like RSV the redox balance can be tweaked such that H_2O_2 mediated death is inhibited. These findings could have potential implications for favorably tailoring the tumor cells to chemotherapy by engaging death proteins and simultaneously switching the levels of ROS from survival (O_2^-) to death mode (H_2O_2).

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ABBREVIATIONS

AIF- Apoptosis inducing factor

Bax- Bcl-2 activated X protein

Bcl-2- B-cell lymphoma 2

Cat- Catalase

C1- Merodantoin

C2- Merocil

Cyclohex- Cycloheximide

Cyt C- Cytochrome C

DCFDA- Dichlorofluorescin diacetate

DioC_6- 3.3 diheyloacarbocyanine iodide

DMSO- Dimethyl-sulphoxide

DPI- Diphenyliodonium

DNR- Daunorubicin

FADD- Fas associated death domain

FBS- Foetal bovine serum

H_2O_2- Hydrogen peroxide

HPLC- High performance liquid chromatography

MC540- Merocyanine-540

MFI- Mean fluorescent intensity

MnSOD- Manganese superoxide dismutase

MTT- 3-(4, 5 dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NHE- Na^+/H^+ exchanger

O_2^- - Superoxide anion

PARP- Poly-ADP ribose polymerase

PBS- Phosphate buffer saline

PDT- Photodynamic therapy

P/I- Propidium Iodide

ROS- Reactive Oxygen Species

RSV- Resveratrol

SDS- Sodium dodecyl sulphate

SOD- Superoxide Dismutase

TBS- Tris Buffer Saline (T) - Tween 20

VCR- Vincristine

VDAC- Voltage gated anion channel

Vit C- Vitamin C

zvad- General caspase inhibitor

$\Delta\Psi m$- Transmembrane potential

1. INTRODUCTION

1.1 Apoptosis in cancer biology- cross talk between mitochondria and caspases:

Cancer is one the leading killers in the world and is caused by building up of genetic alterations which provide them with a selective growth and survival advantage on normal cells. Cancerous cells are thus unable to experience apoptosis driven natural cell death process and major obstacle in modern cancer medicine is still to find the successful treatment for cancer.

Over the past two decades research in the field of apoptosis has flourished and many avenues related to this form of death are being explored. Defined as "programmed cell death" by many, the best and most catching definition of apoptosis being described as " a highly orchestrated form of cell death in which cells neatly commit suicide by chopping themselves into membrane package bits." (MarxJ,1998) Apoptosis is an essential mode of cell death in normal development and is responsible for maintenance of homeostasis of normal cells (Vaux and Korsmeyer, 1999). Typical characteristics of apoptosis include loss of membrane symmetry with phosphatidyl serine externalization to the outer membrane, caspase activation, condensation of cytoplasm and nucleus, internucleosomal cleavage of DNA (resulting in apoptotic bodies) and most dramatically engulfment of these bodies by neighbouring cells (Fadeel et al., 2004). Formation of digits during human embryonic development (Danial and Korsmeyer, 2004) or destruction of unnecessary synaptic neurons are classical examples of apoptosis in developmental biology, while transplant rejection,

autoimmune disorders and Alzheimer's disease are some pathological examples (Popescu and Ankarcrona, 2004).

Cancer can be defined as dysregulation of proliferation or cells that attain a defect in apoptosis.

Molecular research in the field of cancer has welcomed the phenomenon of apoptosis, and uptil today the search for the cure for cancer continues with every year a new dimension being added to the concept of apoptosis (Meier and Silke, 2003) . The diversity of different cancer cell types has made life difficult for researchers to find a common or central mechanism that can be specifically targeted for the treatment of cancer.

Caspases are a family of proteins that play an important role as effectors of apoptosis. The caspases are a group of cysteine proteases that exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis (Donepudi and Grutter, 2002). One caspase can activate another caspase leading to the formation of a caspase cascade that amplifies the death signal.

In a typical cell undergoing apoptosis two distinct mechanisms have been widely described. Intracellular stress signals that involve the Bcl-2 family of proteins at the mitochondria are known to undergo Type II cell death and the other is the death receptor pathway that is independent of the mitochondria and is classified as Type 1

cell death (Scaffidi et al., 1998a). Upon a death signal that engages cell surface receptors like CD95, will lead to recruitment of upstream caspases like caspase 8 via proteins containing death effector domains (DEDs) like FADD and forming a death inducing signaling complex (DISC). Once caspase 8 is activated this will lead to the further recruitment of downstream executioner caspases like caspase 3 or caspase 6 or engage the mitochondria by cleaving Bid (Crompton, 2000; Desagher et al., 1999). Cleaved Bid will then translocate to the mitochondria either independently or serve as a chaperone to move pro-apoptotic molecules like Bax where they will induce the release of cytochrome C (Strasser et al., 2000). Cytochrome C then complexes with Apaf-1 in the presence of caspase 9 to form the apoptosome (Arnoult et al., 2002). The apoptosome will then activate down stream executioner caspase 3. The activation of such caspases will eventually result in the chewing of DNA repair proteins like PARP that are important for normal cellular function (Hengartner, 2000). The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA presiding in the nucleus.

PARP, a DNA repair enzyme is a classical substrate for caspase 3 and its cleavage from 116kd to 86kd denotes that cells have entered a point of no return, and are deemed to undergo apoptosis (Strasser et al., 2000; Wilson, 1998). **(Fig-1)**

APOPTOTIC PATHWAYS

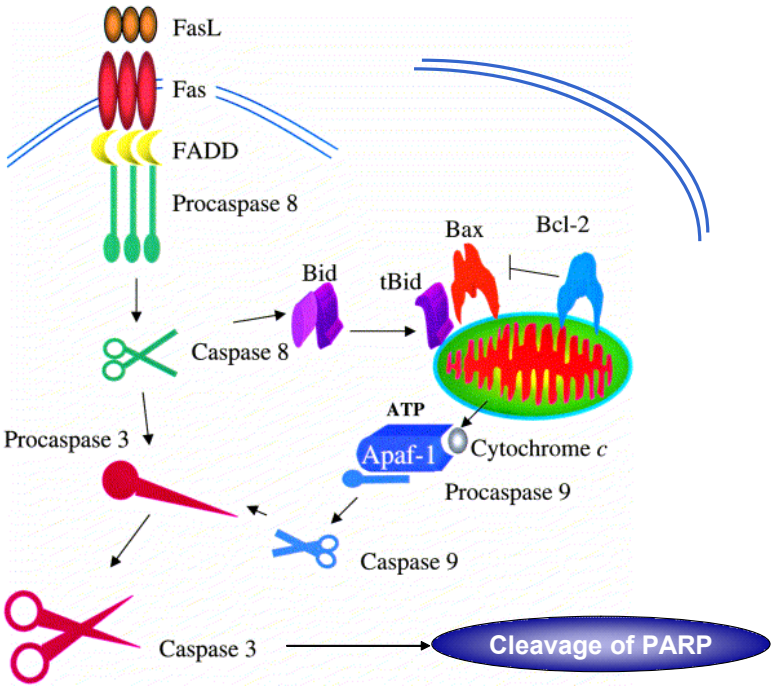

Figure-1 Apoptotic pathways in cancer cell death

Two distinct pathways have been hypothesized resulting in the induction of apoptosis. A receptor pathway that recruits caspase 8 leading to Bid cleavage that engages the mitochondria by release of cytochrome C and caspase 9, or caspase 8 directly activating caspase 3 (by passing the mitochondria). Caspase 3 once activated will execute the destruction of DNA repair enzymes such as PARP. However, drugs can directly activated pro-apoptotic molecules like Bax resulting in direct engagement of the mitochondria.

1.2 Regulation of apoptotic signaling by Reactive Oxygen Species:

Reactive oxygen species are involved in cellular mechanisms such as proliferation and cell death. They are generated physiologically during cell metabolism by reduction of molecular oxygen to produce ROS such as O_2^- and non-radical oxygen species such as H_2O_2. The O_2^- produced can also undergo dismuation to produce H_2O_2. H_2O_2, is weak oxidizing and reducing agent that reacts poorly at physiological concentration but by far is the most stable of the reactive oxygen species (Halliwell and Gutteridge, 1999). During normal cellular function ROS is produced constantly in mammalian cells. They are generated in the mitochondria as a by-product of normal respiration and in other locations as a function of biochemical reactions using oxygen. For example, the smooth endoplasmic reticulum generates ROS through reactions involving cytochrome P450 (Burdon et al., 1990). Another ROS system is well characterized in cell membranes of phagocytic cells that reduce molecular oxygen. In this aspect, the cell membrane bound NADPH oxidase system that recruits Rac, a small GTP binding protein that is a downstream effector of the oncogene product p21-ras and plays a vital role in actin polymerization, Jun kinase activation and most importantly O_2^- production. In non-phagocytic cells proteins that are responsible in O_2^- liberation are not well understood but were shown to be regulated by Rac1(Diekmann et al., 1994; Pervaiz et al., 2001). To describe the functions of Rac in detail is beyond the scope of this thesis. However, just the generation of O_2^- will be taken into account as it constitutes an important part of our hypothesis. The NADPH dependent production of O_2^- can be pharmacologically inhibited using DPI

and will be used later in our experiments. It is also important to be noted that Rac mutants have already been generated in collaboration with Dr. Clement's lab and scientific findings on RacGTPase have already been established (Pervaiz et al., 2001). I have used one of these mutants, and transfection of cells with the dominant negative mutant of Rac (Rac N17) sensitizes cells to apoptosis by lowering the O_2^- levels. The concentration of these free radical species produced within the cell is constantly regulated by the anti-oxidant defense systems within the cell that comprise of enzymes such as SOD (MnSOD in the mitochondria and Cu/Zn SOD in the cytoplasm, nucleus and lysosomes), catalase (located primarily in peroxisomes) and glutathione peroxidase (found in all sub-cellular compartments). These enzymes detoxify H_2O_2 into O_2^- and H_2O. "A balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation, and adaptation to diverse growth conditions." (Nordberg and Arner, 2001) ROS generated have different chemical properties eg:- the hydroxyl radical is highly reactive and reacts immediately with the cellular components and thus is enable to reach distant subcellular targets. On the other hand, ROS species like H_2O_2 or nitric oxide are much less reactive and are able to diffuse throughout the cell (Oberley, 2002). There is now substantial data labeling ROS as oxidative stress inducers and they are often described as damaging and toxic (von Harsdorf et al., 1999). It is generally accepted that, under pathological circumstances, ROS elicit an attack on cellular components by oxidation.

In a nut shell, ROS production is to be tightly regulated by scavengers of ROS to prolong survival of any cell.

1.3 Mitochondria, Bcl-2 family and apoptotic execution:

The Bcl-2 members are a family of proteins that are involved in determining the fate of a cell to undergo apoptosis. The key player in this programmed cell death emerged when Bcl-2, the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death rather promoting cell proliferation (Adams and Cory, 2001). Some of these proteins (such as Bcl-2 and Bcl-XL) are anti-apoptotic, while others (such as Bid, Bad or Bax) are pro-apoptotic. All contain at least one of the four conserved regions known as the Bcl-2 homology domains (BH1-BH4). The death promoters like Bax or Bak contain BH1, BH2 and BH3 domains (Harris and Johnson, 2001; Zong et al., 2001). The sensitivity of cells to apoptotic triggers can depend on the balance of pro- and anti-apoptotic Bcl-2 family of proteins. The battle for survival or death hangs in balance to the effect of these death and survival proteins.

Mitochondria are the energy generating "power houses" of the cell. The organelles play a vital role in redox and pH homeostasis. Apoptosis in response to cellular stresses like UV radiation, CD95, ganciclovir partially involve the receptor mediated cell death and strong data have emerged using receptor knockouts and antagonistic anti-CD95 antibodies to demonstrate that mitochondria can be directly implicated by drugs (Debatin et al., 2002).

Since mitochondria of cancer cells are heavily guarded by survival proteins like Bcl-2 it helps them thrive better than normal cells by protecting them from invasion of death proteins like Bax. Thereby, mitochondria as an organelle becomes a prime

target for novel drugs and mitochondrial apoptosis has been an area of focus by our group and many other groups (Costantini et al., 2000). Although the series of events involved in death receptor-induced sequential activation of caspases has been clearly demonstrated (Krammer, 2000; Scaffidi et al., 1998b), the early signals that trigger caspase recruitment and engagement of the mitochondrial death pathway during non-receptor/drug induced apoptosis are not optimally understood. Death signals induced by drugs engage Bcl-2 family of proteins that will ultimately lead to mitochondrial dysfunction (Green and Reed, 1998). A well known example is that of Bax, a 21kD pro-apoptotic molecule by far the most important apoptotic inducer that predominantly resides in the cytosol and upon a death signal translocates to the mitochondria to destabilize its membrane potential resulting in the release of cytochrome C (Jia et al., 2001; Putcha et al., 1999). Translocation of Bax requires a conformational change unmasking its N-terminus. Bax tends to oligomerize to form Bax-Bax dimers which has been well established by cross linking experiments and play a crucial role in apoptosis (Zong et al., 2001).

Researchers are still arguing as to whether Bax in the mitochondria forms a channel in the mitochondrial membrane (Pavlov et al., 2001) by itself (as its has been shown in some systems like yeast) or interacts with the mitochondrial pore forming proteins like VDAC (Tsujimoto and Shimizu, 2000) or just physically ruptures the mitochondrial membranes to spill out the cytochrome C into the apoptotic system (Crompton, 2000; Zamzami and Kroemer, 2001). Furthermore, disruption of mitochondrial electron transport chain will result in increased production of ROS from the mitochondria and cytosolic acidification will lead to an amplification of the

death signal by recruiting caspases such as caspase 9 and the release of cytochorome C (Desagher and Martinou, 2000) Recently, studies have shown that mitochondria can be directly implicated by anti-cancer agents like doxorubicin and are indeed a rich source of intracellular ROS derived from the mitochondrial respiratory chain during drug-induced apoptosis (Childs et al., 2002; Quillet-Mary et al., 1997)

ROS mediates apoptosis in many cell types. The relationship between ROS and Bcl-2 family of proteins is clouded. Recent reports have substantiated that ROS can sensitize cancer cells to down regulate Bcl-2 expression and render the cells to die by apoptosis (Hildeman et al., 2003). The Bcl-2 expression in mitochondria and in other organelles such as endoplasmic reticulum thus acts as a shield from various apoptotic insults including H_2O_2 (Distelhorst et al., 1996). I was particulary interested in investigating the involvement of the pro-apoptoic Bcl-2 family protein Bax in H_2O_2 mediated apoptosis, using exogenous H_2O_2 or H_2O_2 production triggered by drug exposure.

1.4 A permissive intra-cellular milieu for apoptotic signaling:

According to our hypothesis a slight increase in O_2^- production by mammalian cells (of interest being tumor cells) creates a pro-oxidant state with a pH in a slightly alkaline range that is beneficial for cell proliferation either by mechanisms promoting cell division or those that impede the apoptotic execution signal (Clement and Pervaiz, 2001; Clement and Stamenkovic, 1996).

"Pro-oxidant states can be caused by different classes of agents, including hyperbaric oxygen, radiation, xenobiotic metabolites and Fenton-type reagents, modulators of the cytochrome P-450 electron-transport chain, peroxisome proliferators, inhibitors of the antioxidant defense, and membrane-active agents."(Cerutti, 1985)

These slight increased levels of O_2^- modulate expression of growth genes by DNA alteration or epigenetic mechanisms and perform physiological functions that are beneficial for cell survival. The critical determinant that decides the fate of the cell is the intracellular production of H_2O_2 and the intra-cellular ratio O_2^- and H_2O_2. A lethal shift in particular by drugs that overcomes this pro-oxidant levels offered by O_2^- and generate more H_2O_2, eventually overcome the antioxidant defense systems of tumor cells. In addition, the "decisive tilt" in favor of H_2O_2 creates an environment (acidic milieu) permissive for execution of caspases (Pervaiz and Clement, 2002b). It is important to note that overwhelming the cells with H_2O_2, (> 500uM) will results in necrosis a non-specific form of death as described above (Clement et al., 1998b).

The exact mechanism of H_2O_2 induced apoptosis is unclear and whether its production critically involves the Bcl-2 family of proteins remains clouded. This is

primarily due to the numerous downstream targets of the agent and perhaps because of its divergent signaling. However, it is well understood that exposure of tumor cells to apoptosis inducing concentrations of H_2O_2 (100-500uM) leads to a decrease in O_2^- production subsequently switching the alkaline pH of tumor cells to an acidic range and thereby rendering reduction of the intra-cellular environment. Intracellular acidification has been associated with the Na+/H+ exchanger on the cell membrane that is present in a variety of cell types and its dysfunction is perhaps mediated by PARP activation (Hirpara et al., 2001). Exogenous addition or production of H_2O_2 within the cell will rapidly decrease cellular NAD+ and subsequently ATP levels. Since the exchanger requires phosphorylation that causes conformational change in the channel and relies on the ATP for exchange of sodium to inside and hydrogen ion to outside, depleting the levels will result in failure of this pump (Wu et al., 2003; Yao et al., 2002) . This will result in ineffective extrusion of H+ ions and increasing concentration of H+ ions denotes acidic pH. This is critical in execution of death signal by caspases and is described by us as reductive rather than oxidative stress as illustrated in **(Fig-2)** (Clement et al., 1998b; Hirpara et al., 2001). There is accumulating evidence that ROS can act as signaling molecules, if not all, but in many scenarios of apoptosis. Signalling by H_2O_2 may not appear to be random, as previously assumed but targeted at specific metabolic substrates and components of signal transduction in the apoptotic machinery. The view that ROS are not just toxic by-products but are truly signaling molecules has been further substantiated by the findings that cellular anti-oxidants such as glutathione and thioredoxin not only regulate ROS but also act as "reversible redox modifiers of enzyme function."

(Carmody and Cotter, 2001). Thus, a critical balance exists between O_2^- and H_2O_2 that not only maintains the integrity but also determines fate of a tumor cell.

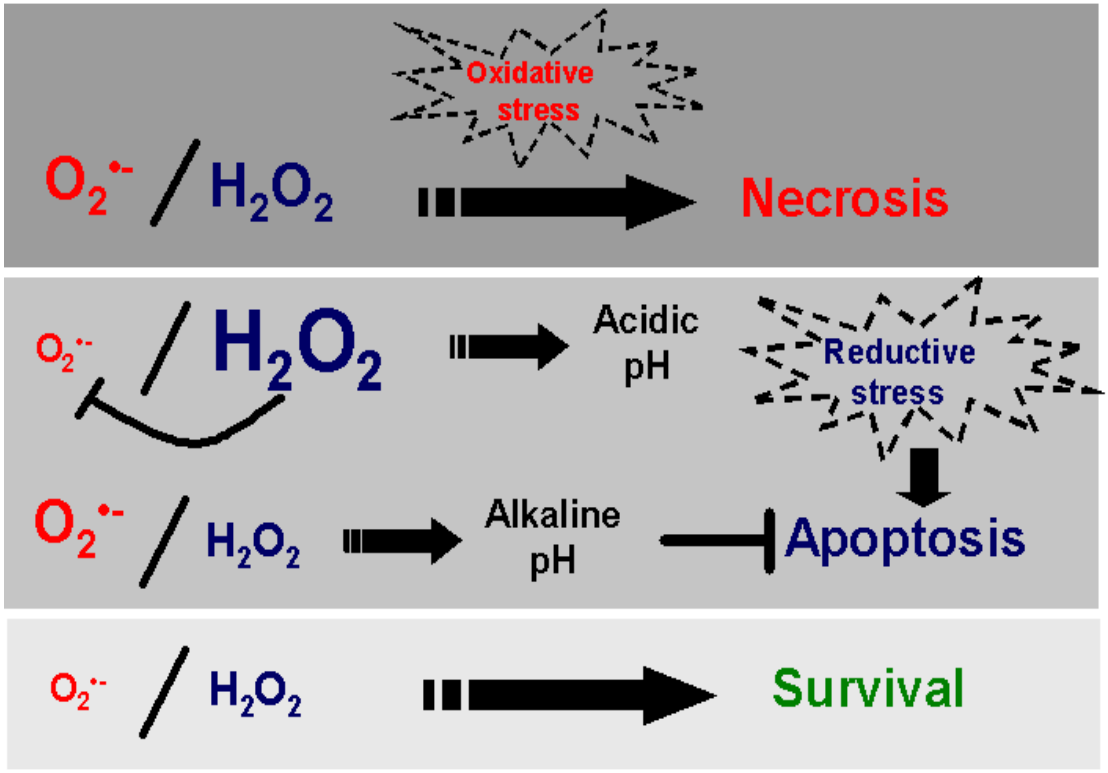

Figure-2: Clement, MV. and Pervaiz, S. Intracellular superoxide and hydrogen peroxide concentration: a critical balance that determines cell survival or death. ***Redox Report (2001)***

This hypothesis is strongly tested in the thesis. Production of hydrogen peroxide has an inhibitory effect on superoxide anion production leading to an acidic pH that render cancer cells to die by apoptosis. This form of cell death is via reductive stress unlike oxidative stress that can overwhelm the system and induce a non-specific form of cell death (necrosis). Contrarily, slight pro-oxidant state gives tumor cells a survival advantage.

1.5 Importance of ROS in generation of novel compounds by photo-oxidation:

Merocyanine-540 (MC540) is a lipophillic dye has been used in several pre-clinical models including extracorporeal purging of tumor cells from autologous bone marrow grafts and as part of conventional photodynamic therapy (PDT) a modality used in the treatment of not only solid tumors but also in diseases like atherosclerosis, age-related macular degeneration and rheumatoid arthritis (Kubo and Sieber, 1997; Pervaiz, 2001). A major disadvantage of conventional photodynamic therapy or its limitation lies in the fact that photosensitizer and light both are required to observe any damaging effects on the target. This problem has been circumvented by a noval modality known as "*pre-activation"*, a method that can generate anti-tumor agents/bio-active compounds by controlled illumination of chromphores prior to their use in any biological systems (Franck and Schneider, 1992; Gulliya et al., 1992).

The apoptosis inducing ability of vitamin C has been described by many others and its anti-oxidant capabilities have been established (Connelly et al., 2003; Kang et al., 2003). Vitamin C is an important water soluble vitamin that is essential for collagen, carnitine and neurotransmitters biosynthesis. Most plants and animals synthesize vitamin C for their own requirement, unlike apes and humans can not synthesize vitamin C due to lack of an enzyme gulonolactone oxidase. Hence, vitamin C has to be supplemented mainly through fruits, vegetables and tablets. Many health benefits have been attributed to Vitamin C such as antioxidant, anti-atherogenic, anti-

carcinogenic, immunomodulator and its unknown role in cold prevention. The debate continues on the use of mega doses of Vitamin C. Does vitamin C act as an antioxidant or pro-oxidant; is it a cause cancer or does it interferes with cancer therapy? Questions to these aspects are still being addressed and role of vitamin C in cancer therapy is controversial (Naidu, 2003).

In light of the findings on the beneficial role of vitamin C on photofrin we designed this study to elucidate the effect of ascorbate on photo-oxidation of MC540 as an adjunct to MC540 in PDT or its pre-activation together in the presence of MC540 using human leukemia cells (Kelley et al., 1997). The reason for the use of vitamin C in combination with MC540 was the fact that during the process of photo-oxidation the central polymethine chain is attacked by oxygen resulting in the generation of intermediate compounds. Such a destruction of a photo-sensitizer is rare and perhaps exclusive to MC540. The by products of this compound have been purified and are undergoing thorough investigation as apoptosis inducing molecules in our laboratory. I wanted to observe whether vitamin C had any role on the stability of these molecules or whether vitamin C hinders the photo-oxidation of MC540. Data generated in our laboratory reveals that purified photo-products of MC540 (Pervaiz et al., 1999b), merodantoin (C1) **(Fig-3)** and merocil (C2) are powerful bio-active compounds (Pervaiz et al., 1998) that have anti-tumor activity against a variety of tumor lines (Sharma and Gulliya, 1995). A brief introduction to MC540 was worth mentioning as part of this thesis. These compounds liberate intracellular H_2O_2 and are wonderful tools to study apoptosis and redox regulation in tumor cells. Furthermore, signal transduction induced by C1 and C2 is H_2O_2 mediated and recently work on C2

has been published to demonstrate that it induces “intracellular acidification triggered by mitochondrial derived H_2O_2 production that is an effector mechanism for drug induced apoptosis in tumor cells.” (Hirpara et al., 2001)

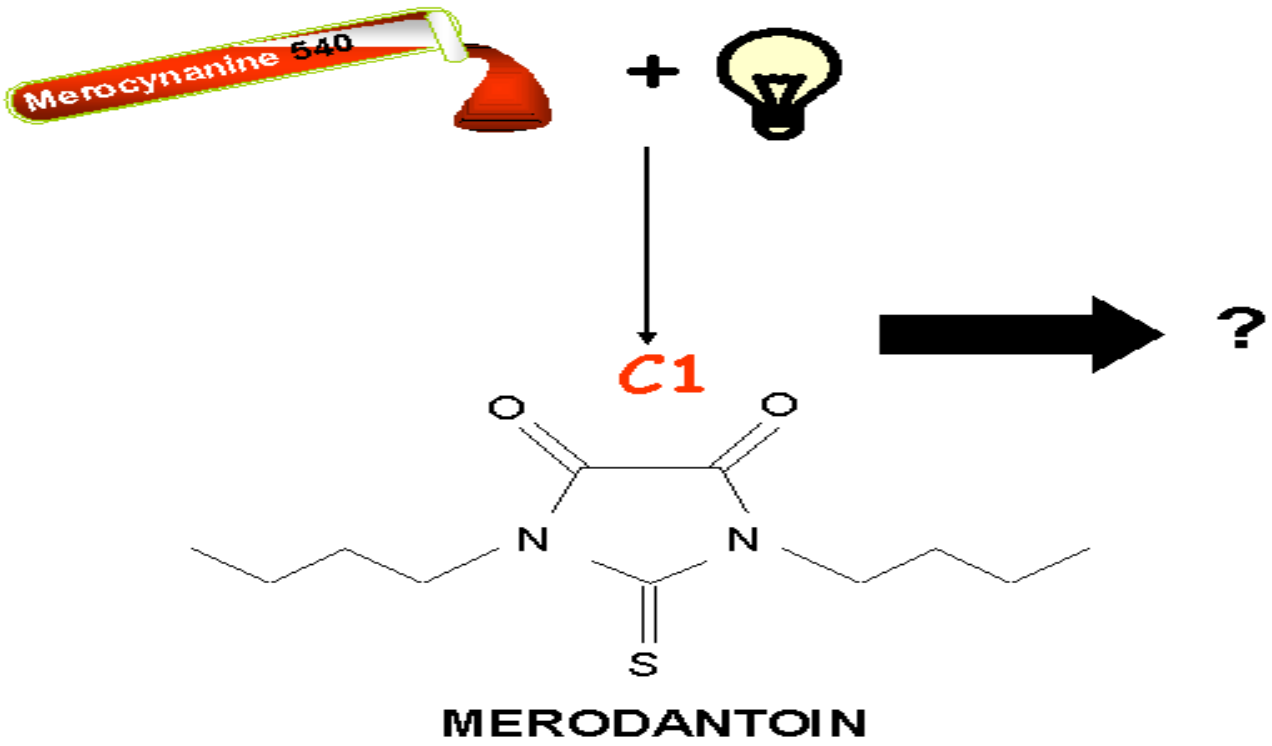

Figure-3: Photo-activation of parent compound MC540 under light generates novel bio-active molecules like C1. Mechanism of action is still unknown.

The thesis will provide an insight into the mechanism of action of C1 on tumor cells and will be used in studying our redox hypothesis. The purpose of using C1 was to derive information on its mechanism that has not been reported previously.
The compound is diversified and may hold a promising future in anti-cancer therapy.

1.6. Role of intra-cellular redox status on tumor cell response to combination chemotherapy:

The current trend in modern cancer medicine is the use of chemotherapeutic agents in various combinations. One area of recent interest in cancer biology particularly combined chemotherapy is the chemopreventive potential of natural products. Among the compounds being evaluated for their cancer inhibiting activity is a phytoalexin, resveratrol (RSV) a trans-3,5,4 trihydroxystilbene, found in mulberries, peanuts, grapes and wines derived from grape products. It is known for its diverse biological activities in cellular proliferation, induction of mitochondrial and death receptor induced apoptosis, anti-inflammatory effects involving cytokines, inhibition of androgenic and estrogen receptor activity and in context of this thesis its anti-oxidant property (Bhat et al., 2001; Pervaiz, 2003; Soleas et al., 2001). The agent is classified as a class of phenolic compounds and broadly is considered as a flavanoid or placed in the category of plant antibiotics (Pervaiz, 2003). Phenolic compounds are responsible for colour and give a bitter taste to wines. RSV is known to occur in free and glycosidically bound form, which is called piceid or polydatin. It has been proved as remarkably potent at preventing experimental skin tumours in mice and at inhibiting the replication in vitro of human leukemia cells. Several studies showed the inhibition of low density lipoprotein (LDL) oxidation and lipid peroxidation in vitro, thereby decreasing the risk of coronary artery disease.

Interesting data has emerged demonstrating that consumption of French wine decreased the incidence of coronary artery disease (The French Paradox). Researchers have clearly demonstrated including our group, that the chemopreventive activity of

RSV could be due to its ability to induce apoptosis in human leukemia, breast carcinoma cells and pancreatic carcinoma cells (Clement et al., 1998a; Ding and Adrian, 2002; Tinhofer et al., 2001). However, depending upon the cell type and the concentration used, RSV has been shown to induce or inhibit cellular proliferation and death signaling (Ahmad et al., 2001; Clement et al., 1998a; Huang et al., 1999; Jang et al., 1997; Joe et al., 2002; Mizutani et al., 1998; She et al., 2003)

Reports have also shown the role of RSV in inducing oxidative stress leading to apoptosis but its protection on cancer cells against oxidative stress has been conflicting. Moreover, the mechanisms governing these responses are unclear. Indeed, many have reported that RSV may act as an anti-oxidant by scavenging H_2O_2 (De Salvia et al., 2002). This thesis will discuss the role of RSV on H_2O_2 signaling by using exogenous H_2O_2 or using chemotherapeutic agents that may signal via H_2O_2 production.

Initially, I was first interested in looking at the effect of RSV as an adjunct to one of the novel isolated anti-cancer compounds C2 derived from MC540 or its use in combination with known chemotherapeutic agents like vincristine (VCR) and daunorubicin (DNR).

Vincristine, a vinca alkaloid is extracted from the flower of the Madagascar periwinkle (*Catharanthus roseus*) (Huschtscha, 1996). VCR binds to the microtubular proteins of the mitotic spindles at metaphase, leading to the crystallization of the microtubules and mitotic arrest, eventually leading to DNA fragmentation (Groninger et al., 2002). DNR is isolated from the fungi *Streptomyces caeruleorubidus*.

Daunorubicin is an anthracycline antibiotic which damages DNA by intercalating with its anthracycline portion. It inhibits DNA polymerases and affects regulation of gene expression (Kim et al., 2003). Both agents are being used in modern medicine today. This thesis also provides evidence that vincristine and daunorubicin may have a redox component that is responsible for DNA fragmentation within the cell, and some mode of death induced by these drugs could be ROS related.

In light of the above the overall goal was to assess the role of intracellular H_2O_2 in death signaling. Using three different systems we questioned the mechanism of H_2O_2 as follows:

1. In the generation of photo-oxidized novel molecules and to study the effect of of vitamin C in this process.
2. Investigate the role of H_2O_2 and drug induced production of H_2O_2 on mitochondrial engagement during drug apoptosis in particular Bax recruitment.
3. Using a combination chemotherapy model to assess the effect on intra-cellular milieu on H_2O_2 mediated tumor cell apoptosis.

These have been described in detail in the section on "Aims" **(3)**

2. MATERIALS and METHODS

2.1 Tumor Cell Lines and Cell culture:

The cells were cultured with supplements, specified in **Table-1**. Cells were grown and passaged in a 37ºC incubator with 5% CO_2 partial pressure. The morphology of the cells was observed daily under a light microscope to ensure their health status before subsequently using them for experiments.

The human promyelocytic leukemia (HL-60) cell lines were purchased from ATCC (Rockville, MD). CEM human leukemia cell lines were generously provided by Dr. Roberta A. Gottlieb, Scripps Cancer Center, La Jolla, CA, USA.

The human colon carcinoma cell line HCT116 Bax +/- and Bax -/- was a generous gift from Dr.Vogelstein at Johns Hopkins University, Baltimore (USA). Cell line was cultured in McCoy's supplemented with 10% FBS, 1% L-Glutamine, 1% S-Penicillin.

Table 1: Growth supplements for tumor cell lines

Cell lines	FBS	Culture medium	Standard antibiotics	L-glutamine	Other requirements	Morphology
HL-60	10%	RPMI 1640	Penicillin-Streptomycin	1%	-	myeloblastic
CEM	5%				-	lymphoblast
HCT116		McCoy's			-	epithelial

2.2 Chemicals:

- From *Bio-rad Laboratories Inc. (Hercules, CA)* the following was purchased: caspase 3 (DEVD-AFC) and 9 (LEHD-AFC) fluorescent assay kit, general caspase inhibitor z-vad-fmk
- From *Calbiochem, (San Diego, CA)* the following was purchased: DAG kinase assay.
- From *Duchefa Biochemie B.V. (Haarlem, Netherlands)* the following was purchased: Tween 20.
- From *Eastman Kodak Co. (Rochester, NY)* the following was purchased: Kodak BioMax films.
- From *Gibco-BRL Inc. (Gaithersburg, MD)* the following were purchased: FBS, L-Glutamine and Streptomycin-Penicillin antibiotics.
- From *HyClone Inc. (South Logan, Utah)*, the following was purchased: culture medium RPMI 1640.
- From *ICN Pharmaceuticals Inc. (Aurora, Ohio)* the following was purchased: vincristine.
- From *Merck Inc, (Whitehouse Station, NJ)* the following were purchased: sodium citrate and methanol.
- From *Molecular Probes Inc. (Eugene, OR)* the following was purchased: CM-H_2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorofluorescin-diacetate) dye, mito-tracker.
- From *NUMI Media Preparation Facility (NUS, Singapore)* the following were purchased: 10x PBS, and 10x SDS.

- From *Pall Life Sciences Co. (Ann Arbor, MI)* the following was purchased: PVDF (polyvinylidene fluoride) membrane.
- From *Pharmingen Co. (San Diego, CA)* the following was purchased: mouse monoclonal anti-PARP/Bax/Bcl-2/Cytochrome C/Anti-CD95 primary antibody.
- From *Pierce Chemical Co. (Rockford, IL)* the following were purchased: Commassie blue solution, goat anti-mouse IgG HRP conjugated secondary antibody, Restore stripping buffer and SuperSignal West Pico chemiluminescent kit.
- From *Sigma-Aldrich Co. (St. Louis, MO)* the following were purchased: Merocyanine 540 (MC540), vitamin C, absolute ethanol, aprotinine, BCECF-AM (2',7'-bis(2-carboxyethyl)-5,6-carboxyfluoresce acetoxymethyl ester) dye, β-mercaptoethanol. bromphenol blue, BSA, CICCP (carbonyl cyanide m-chlorophenyl-hydrazone), crystal violet, daunorubicin, $DiOC_6$ (3,3' dihexyloxacarbocyanine iodide), DMSO, DPI (diphenyleneiodonium), DTT, EDTA, glycine, HBSS (Hank's balanced salts), hydrogen peroxide (H_2O_2), lucigenin (bis-N-methylacridinium nitrate), MTT (3-[4,5-dimethyl-2-hiazolyl]-2,5-diphenyl tetrazolium bromide), pepststin, PI (propidium iodide), $Mgcl_2$ percoll, nigericin, resveratrol, RNAse A, SDS, sodium pyruvate, somatic cell ATP releasing reagent, Trifluoroacetic acid (TFA), Trypan Blue exclusion dye, Urea
- From *Whatman Inc. (Ann Arbor, MI)* the following was purchased: blotting paper Culture media RPMI were purchased from Hyclone laboratories while FBS, L-Glutamine, S-Penicillin and McCoy's 5A were purchased from GIBCO-BRL, Gaithersburg, MD.

- *From Millipore, MA, USA*)-The water used in the mobile phase was Milli-Q grade
- *From Tropix*- Galactostar mammalian transfection kit.
- *From Qiagen*- Superfect transfection reagents.
- *From Fishcer Scientific USA*- HPLC grade methanol and acetronitrite

2.3 Solutions and Buffers:

- Acridine Orange was made in 1xPBS as a 10mg/ml stock solution and stored at 4^0C.
- Buffer A was prepared as 0.3M sucrose, 5mM TES, 0.2mM EGTA, pH 7.2 with KOH and stored at 4°C.
- Catalase was prepared as 50, 000 units stock in plain sterile RPMI.
- Cell lysis buffer- ordered and used as recommended by the vendor.
- Crystal Violet was prepared as a 0.75% solution in 50% ethanol to distilled water with 1.75% formaldehyde and 0.25% NaCl and stored at room temperature.
- Cycloheximide was made in RPMI 1640 as a 100mg/ml solution and kept at 4^0C.
- DPI was dissolved in DMSO to a stock concentration of 50mM and stored at 4°C.
- Ethanol 70% was diluted from absolute ethanol in distilled water and kept at room temperature.
- Ethidium Bromide was made in 1XPBS at 10mg/ml and stored ar4^0C.
- Euk-8 was prepared as 20mM stock in plain sterile RPMI.
- Extraction buffer was prepared as 200mM mannitol, 68mM sucrose, 50mM pipes-KOH pH 7.4, 50mM KCL, 5mM EGTA, 2mM $Mgcl_2$, 1mM DTT, and protease inhibitors.

- General caspase inhibitor (zvad)- was prepared as 20mM stock in 100% sterile DMSO and stored at -20°C.
- HBSS was prepared as 9.8g/L HBSS and 0.35g/L sodium bicarbonate in distilled water and stored at 4°C.
- High K^+ buffer was prepared as 135mM KH_2PO_4 and 20mM NaCl in distilled water and stored at 4°C.
- Low K^+ buffer was prepared as 110mM KH_2PO_4 with 20mM NaCl in distilled water and stored at 4°C.
- Loading buffer was prepared as 62.5mM Tris-HCL (pH 6.8), 6M Urea, 10% Glycerol, 2% SDS, 0.00125 % Bromphenol blue and 5% β-mercaptoethanol and stored at -20°C.
- Lucigenin was freshly dissolved in distilled water to a stock concentration of 125mg/mL.
- Mannitol was preared as 1mM stock in plain sterile RPMI.
- MC540 was made in 70% ethanol:dd water at 1 mg/ml and stored in the dark at 4^0C
- MTT was freshly dissolved in RPMI 1640 to a stock concentration of 4mg/mL.
- PARP lysis buffer was prepared as 50mM Tris HCl pH6.8, 6M urea, 3% SDS, 0.003% bromophenol blue, 6% β-mercaptoethanol and stored at 4°C.
- PBS (1x) was diluted from 10x PBS in distilled water and kept at room temperature.
- PI stock solution (50x) was dissolved in sodium citrate buffer to a stock concentration of 0.5mg/mL and stored at 4°C in the dark.

- PI:RNAse A solution was freshly prepared with 1/50 volume of PI and 1/40 volume of RNAse A stock solution in 38mM sodium citrate buffer.
- Protease inhibitors-1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml soybean trypsin inhibitor and 10 µg/ml aprotonin],
- RNAse A was dissolved in 10mM Tris-HCL (pH 7.5) and 15mM Nacl to a stock concentration of 10mg/mL (40x) and stored at -20°C.
- SDS (1x) was diluted from 10x SDS in 1x PBS and kept at room temperature.
- Semi-dry transfer buffer was prepared as 29g Tris base, 14.5g glycine, 18.5mL 10% SDS, 1L methanol in 5L of distilled water and stored at room temperature.
- Sodium citrate buffer was diluted in 1x PBS to a final concentration of 38mM (pH 7.0) and kept at room temperature.
- Somatic cell ATP releasing reagent (1x) was dilluted from 10x somatic cell ATP releasing reagent in absolute ethanol and stored at 4°C.
- Sorenson's glycine buffer was prepared as 0.1M NaCl and 0.1M Glycine (pH 10.5) in distilled water and stored at 4°C.
- Sphinomyelinase inhibitor
- TBS was prepared as 500ml 1M Tris HCl (pH7.4) and 87.6g NaCl in 10L of distilled water and stored at room temperature.
- TBST was prepared as 2L TBS and 1ml of Tween 20 and stored at room temperature.
- Vitamin C was prepared as a stock of 10mg/ml in distilled water.

2.4 Drugs:

- Merocil (C2) and Merodantoin (C1) photo-products derived from MC540 were purified and dissolved in DMSO to prepare a stock of 100mg/ml and stored at -80°C.
- Hydrogen peroxide (30% grade) was dissolved in plan RPMI as a 10mM stock solution and used fresh for every experiment performed.
- Resveratrol was dissolved on DMSO at a stock concentration of 100mM and then subsequent dilution was made at 2mM in plan sterile RPMI to be used in experiments.
- Daunorubicin was prepared in RPMI 1640 as a stock solution of 10mg/ml and kept at 4^0C
- Vitamin C was prepared as 10mg/ml stock in distilled water and stored at 4^0C
- Vincristine was made in RPMI as a 10mg/ml solution and stored at 4^0C.

2.5 Equipments:

The electroblotting unit (Hoefer-SemiPhor Semidry Transfer) was from *Amersham Biosciences Ltd. (Uppsala, Sweden)*, the flow cytometry (Coulter EPICS Elite ESP) together with the WINMDI (Windows multiple document interface for flow cytometery) software was from *Beckman Coulter Inc. (Hialeah, FL)*, the luminometer (TD-20/20) was from *Turner Designs Inc. (Sunnyvale, CA)*, the spectrofluorometer /

automated ELISA reader (Tecan Spectrfluoroplus) was from *Tecan Ltd. (Maennedorf, Switzerland)* the transfer unit was from *Hoefer-SemiPhor*, HPLC system was from *Waters, Miford, USA*, the spectro-photometer was from *SLT Lab Intruments*, *Austria*, and common laboratory apparatus were all obtained from *NUMI Supplies (NUS, Singapore)*.

2.6 Photo-activation of MC540:

MC540 was dissolved in 70% ethanol: distilled water at 1mg/ml. 8-10 petri plates containing 8ml of MC540 solution were exposed to 8 fluorescent lamps (GE cool white, 40W, Cleveland, OH) for 18 hours **(Fig-4)**. After photoactivation, ethanol was evaporated by rotaevaporation (NUMI, CRC, Singapore). Photoactivated MC540 (pMC540) or p(MC540+VitC) was diluted in 2.5% ethanol:1xPBS at a stock concentration of 50mg/ml and stored at -20^0C for 1 month **(Fig-5**).

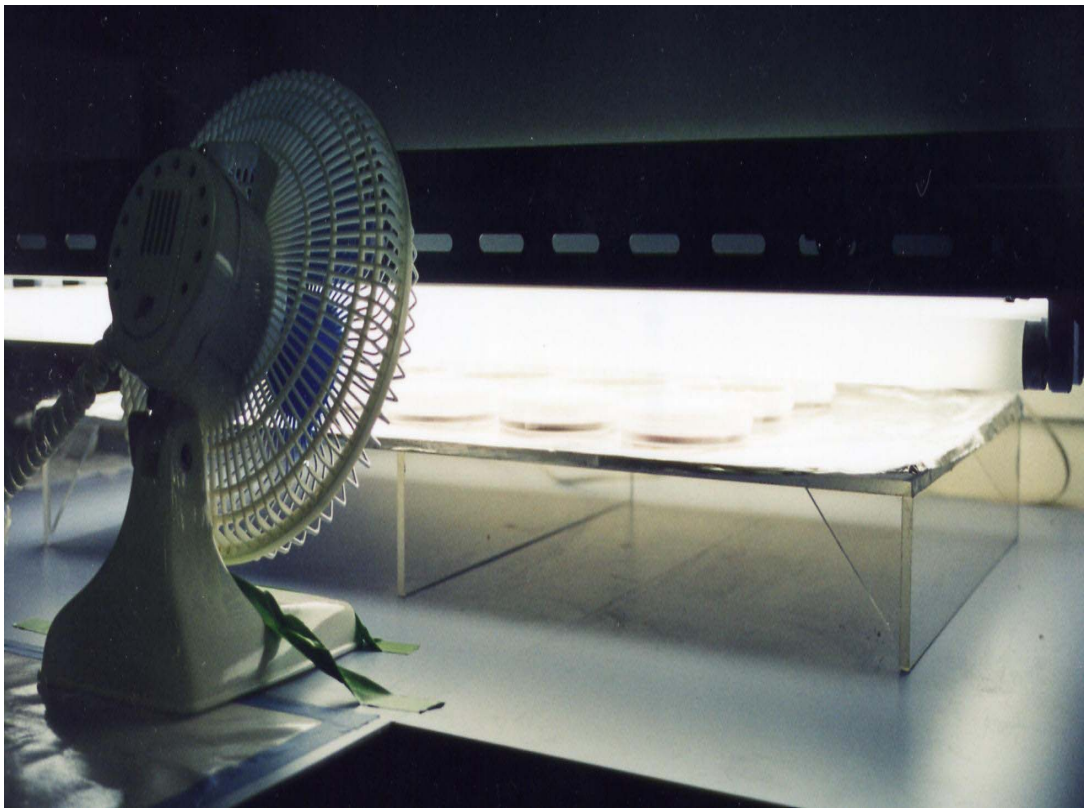

Figure-4: A photo-activation set-up at the Tumor Biology Laboratory.

MC540 as a red lipophillic dye is transferred in sealed petri-dishes and photo-activated under a bank of white tube lights (40W).

Figure-5: MC540 undergoing photo-activation in our laboratory.

MC540 above exists as a dark red lipophillic dye. Activation under light (photo-activation) results in change in colour to light red and finally to a light orange colour indicating activated MC540. It is from this photo-activated mixture compounds C1 and C2 are purified.

2.7 Analysis of vitamin C concentration by HPLC:

Vitamin C concentration was analyzed using a HPLC system equipped with a photodiode array detector (Waters, Milford, MA, USA) and Delta PAK C18 column (15 μm particle size, 100 Å pore size, 3.9 x 300 mm). Gradient elution was started at 98% A and 2% B (solvent A: 0.01% TFA in water; solvent B: 0.01% TFA in acetonitrile). The proportion of solvent B was increased linearly from 2% to 30% in 20 minutes. The flow rate was 0.5 ml/min. The chromatogram was recorded at 245 nm. The concentration of vitamin C in the sample was calculated from the peak area using pure vitamin C as a standard.

2.8 Spectro-photometric analysis*:*

Conversion of parent MC540 to activated MC540 with or without vitamin C was measured by loading samples in cuvettes set at auto zero, with methanol as a standard/reference. Absorption spectrum was recorded as kinetics from 190nm-600nm against Armstrong (A°) using the spectro-photometer (SLT Lab Instruments, Austria).

2.9 Cell Viability Assays:

2.9.1 Trypan Blue exclusion Assay:

10ul of cells treated or untreated were mixed with 10ul of the trypan blue dye and pipetted under coverslip of a Neubar's chamber/haematocytometer. Live cells stained blue where as dead cells were transparent and recored using a hand counter.

2.9.2 MTT assay:

HL-60 cells/CEM (1×10^5 cells/well) in 96 well plate were treated with different doses of vitamin C (100-1000μM) or with pMC540540(100μg/ml), pMC540540+Vit C(100ug/ml+1mM), pMC540540+pVit C (100ug/ml), p(mc+Vit) (100ug/ml) pVit C(100ug/ml), and 50μg/ml of C1, C2, 100um of H_2O_2 (100uM), 1.25ug/ml of vincristine and 0.2ug/ml of daunorubicin. In experiments where pre-incubation for one hour was performed with catalase (1000units), resveratrol (4-8uM), DPI (1.25uM), zvad (100uM), cycloheximide (5-10ug/ml), anti-CD95 (1-3ug/ml), Sphi (1mg/ml) wherever stated in the results. Cytotoxicity was determined by MTT assay. After drug exposure, 50μl MTT from the stock solution was added to each well and incubated for 4 hours at 37^0C in the dark. After 4 hours incubation with MTT the plate was spun at 1200rpm for 5 minutes. Crystals were dissolved in 200 μl DMSO + 10μl Sorenson's glycine buffer after removing the supernatant. Viability was determined by absorbance at 570nm wavelength using an automated ELISA reader.

2.9.3 Crystal Violet assay:

Monolayer cell lines HCT116 (2×10^4 cells/well) were plated in 96 well plates or (2X105) in 24 well plates with the culture medium for 24 hours prior to treatment to allow the cells to adhere to the plates. The medium was then replaced with fresh medium containing different doses of H_2O_2 (100uM-500uM) for different time points (4 hours-18 hours). Crystal violet staining was used to assess cell viability. After treatment, cells were washed with 1x PBS and 50μl of crystal violet solution was added for 15 minutes. Cells were then washed with, air-dried and crystals were

dissolved with 1%SDS in 1xPBS. Viability of cells were determined by using absorbance at 595nm wavelength using an automated ELISA reader. Cell death was calculated as:

$$\frac{\text{Mean of triplicate OD values of cells incubated with the drug}}{\text{Mean of triplicate OD values of cells incubated with the solvent}} \times 100$$

2.10 Analysis of Cell size:

After treatment, cells were washed twice with 1xPBS+1%FBS and pallets were resuspended in 500µl of 1xPBS+1%FBS to determine cell size by flow cytometry. The ability of the cells to scatter light in forward direction (FS) correlates with cell volume, therefore treated cells were analyzed for a loss in the FS as compared to untreated cells. 10,000 events per sample were used for analysis.

2.11 Analysis of DNA fragmentation by PI:

Following exposure of cells to the anti-cancer agent, cells were centrifuged and pellets were resuspended in 500µl of 1xPBS+1%FBS and immediately fixed with 70% ethanol: water. 70% ethanol: water was added while vortexing the cells to avoid clumping. After that, the cells were incubated for 15 minutes on ice and spun at 1000g for 5 minutes. Cells were washed with 1xPBS+1%FBS once and then cells resuspnded in 500µl of PI: RNAse A solution for 30 minutes at 37^0C in the dark. Stained cells were analyzed by flow cytometry with excitation wavelength at 488nm and emission wavelength at 610nm (34). Flow cytometer data were determined by using the WINMDI software.

2.12 Determination of Caspase-3, 8 and -9 activities:

Caspase 3 (CPP-32), caspase-8 and Caspase 9 activities were determined by using the ApoAlert fluorescent Assay kit for caspase-3 and cspase-8. 1×10^6 tumor cells were treated with pMC540540540 (50μg/ml) for different time points (4 hours-18hours) or with 50μg/ml of C1, C2, or H_2O_2 for 12 hours. Treated cells were washed once with 1xPBS. Monolayer cells were washed after detaching with EDTA. Cells pellets were resuspended in 50μl of chilled cell lysis buffer (provided by the supplier) and incubated on ice for 10 minutes. After that, 50μl of 2X reaction buffer with 10mM DTT and 5μl of the conjugate substrate (DEVD-AFC for caspase-3 and IETD-AFC for caspase-8 assay and LEHD-AFC for caspase 9) was added to each sample. Cells were incubated for 60 minutes at 37^0C. Protease activity was determined by relative measuring the relative fluorescence intensity at 505 nm following excitation at 400 nm using a Spectofluorometer (Luminescence Spectrometer LS50B, Perkin Elmer, Burkinghamshire UK).

To inhibit caspase activity, pan-caspase inhibitior zvad-fmk (50uM) was added to the induced samples in 50μl of 2X reaction buffer prior to the addition of the substrate. Subsequently, 5μl substrate was added and then incubated at 37^0C for 60 minutes and protease activity determined as described before.

2.13 Subcellular fractionation by differential centrifugation:

HL-60/HCT116 cells (10-30×10^6) depending upon the protein to be identified were treated with various drugs and washed once in ice chilled phosphate-buffered saline (PBS), resuspended in isotonic buffer A (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5) supplemented with protease inhibitors and incubated on ice for 15-30 mins. Samples were then homogenized using 20-30 strokes in the Dounce homogenizer. Nuclei and unbroken cells were separated at 120 *g* for 5 min as the low speed pellet (P1). This supernatant was centrifuged at 10 000 *g* for 10 min to collect the heavy membrane pellet (HM). This supernatant was centrifuged at 100 000 *g* for 30 min to yield the light membrane pellet (LM) and final soluble cytosolic fraction (S). For Cytochrome C the supernatant was spun at 14,000g for 15 minutes at 4^0C. After centrifugation supernatant was stored at - 80^0C until analysis of cytochrome C was performed by western blotting.

2.14 Immunostaining by confocal microscopy:

HL-60 cells before or after treatment (2-4x10^6) were washed once with 1x PBS and stained with Mitotracker (125nM, Molecular probes) for 30-45 mins in cultural condition. After staining cells were washed twice with 1x PBS and fixed with 3.7% formaldehyde. Cells were then permeabilized by 70% ethanol. Consequently cells were washed and incubated with purified mouse primary antibody of Bax/Cytochrome C (1: 50, Pharmingen) for 1 hr at 4C. After primary staining cells were washed and stained with secondary antibody anti-mouse- FITC (1:100, DAKO)

for 30 mins at 4C. In the end cells were washed twice with 1x PBS and analysed by LSM Zeiss confocal microscope using Rhodamine and FITC filters. In a separate set of experiments, HCT116 cells (2×10^3) were grown on cover slips, exposed to 100 μM of H_2O_2, fixed with methanol:acetone (1:1 vol/vol), and incubated for 2 hours at 37°C with 1:150 dilution (in 3% bovine serum albumin [BSA]) of monoclonal anti-Cyt.C antibody (clone 7H8.2C12)

After three washes with 1× PBS + 1% FBS, cells were exposed to a 1:20 dilution of antimouse FITC-conjugated IgG (Pharmingen) for 1 hour, washed twice, and analyzed by confocal microscopy (NUMI Core Facility, NUS, Singapore).

Bax translocation to the mitochondria was defined by co-localization of green (Bax) and mito-tracker (red) as orange-yellow Bax clusters.

Cytosolic Cyt.C was defined as diffuse cytoplasmic staining as compared with punctate mitochondrial Cyt.C staining obtained with nontreated control cells.

2.15 Anti-CD95 staining:

Briefly, HL60 cells (1×10^6) were treated with C1 (50ug/ml) or hydrogen peroxide (100uM), washed twice with phosphate-buffered saline (PBS) + 1% FBS, and fixed in 1mL of 4% paraformaldehyde for 15 minutes on ice. After a wash with PBS, cells were permeablized in ethanol for 1 hour on ice, washed with PBS, and incubated with 1 μg of anti-CD95 or anti-CD95L for 30 minutes on ice. Following another wash with PBS + 1% FBS, samples were exposed to FITC-conjugated goat anti-mouse IgG for 30 minutes, washed, and resuspended in 0.5 mL of 2% paraformaldehyde. Mouse $IgG_1\kappa$ was used as isotype control. Viable cells were gated on forward side scatter

analysis, and a total of 10,000 events were analyzed by flow cytometry using an emission wavelength of 525 nm.

2.16 Determination of protein expressions by western blotting:

Before western blotting can be carried out to determine the intracellular expression of proteins, the cells were lysed in 1XRIPA or fractions were loaded directly. Protein concentration was detected using Commassie Blue reagent and calculated by plotting a graph against protein standards. For determination of PARP expression by western blotting, HL-60 cells after being subjected to treatment with drugs were washed once with 1x PBS and lysed with PARP lysis buffer (50µL/2x10^6cells) instead.

For the detection of PARP and cleaved PARP fragments by western blotting 10% resolving gel was prepared and poured into sandwiched glass plates, followed by the stacking gel **(Table-2)**. Higher resolving gel is used for more efficient separation of smaller proteins. The resolving gel separates the proteins into different size bands while the stacking gel aligns the protein at the gel interface for better resolution. Next 50µL of the protein extract from the cell lysate was heated for 10min in a heat block set at 100°C. The samples were then subjected to the SDS-PAGE electrophoresis for separation running at 200V, 10mA, 10W for 20h. For Bax, Bcl-2, Cytochrome C samples were run at 120V, 4mA, 10W for 12-14 hours. The gel was then transferred to a PVDF (polyvinylidene difluoride) membrane using the Hoefer-SemiPhor transfer unit, again running at 24V, 320mA, 10W for 2hrs.. For transferring, a PVDF membrane was sandwiched between 3 layers of 3mm blotting paper that had been soaked in semi-dry transfer buffer for 10min. Prior to soaking in semi-dry blot, the PVDF membrane was soaked in methanol for 5min. After the transfer, the membrane

was immediately blocked for 1h with 2.5g non-fat dry milk in 50mL TBST. The membrane was then blotted with mouse monoclonal anti-PARP primary antibody at 1:1000 dilutions, overnight at 4ºC with gentle agitation. This was followed by incubation with 5µL of the goat anti-mouse secondary antibody conjugated with horseradish peroxidase diluted with 1.25g of non-fat dry milk in 25mL TBST for 1h at room temperature with gentle rocking on the shaker. In between each incubation and blocking process it is necessary to rinse the membrane once and wash it three times (15min each) with TBST. Membranes were wrapped in plastic thin wraps and chemiluminescence was detected using SuperSignal West Pico Chemiluminescent kit. The protein expression were then developed on Kodak films. For stripping, 10ml of Restore stripping buffer was used twice at room temperature and then wahed again with TBS to re-probe with fresh antibody.

Table 2: Compositions of resolving and stacking gels for electrophoresis

10% Resolving gel	
Reagents	Volume (ml)
Distilled water	15.9
Polyacrylamide	13.3
Tris at pH 8.8	10.0
Ammonium Persulphate	0.4
TEMED	0.016

Stacking gel	
Reagents	Volume (ml)
Distilled water	10.35
Polyacrylamide	2.49
Tris at pH 6.8	1.89
Ammonium Persulphate	0.17
TEMED	0.015

Antibody dilutions:

Bax-1:2000, Bcl-2 1:2500, PARP- 1:5000, MnSOD- 1:1000, Cytochrome C-1:1000 in TBS.

2.17 Detection of Apoptotic/Necrotic cells by Confocal Microscopy:

HL-60 cells (1×10^6 cells/ml) were treated with 20ug/ml MC540 alone or in combination with 1mM of vitamin C for 15mins -60 mins at 37^0C under PDT and then analyzed by confocal microscopy both with transmitted and after staining with acridine orange and ethidium bromide. Treated cells were washed once with 1X PBS and then resuspended in 100µl of PBS. 1µl of dye mix (100µg/ml of acridine orange + 100µg/ml ethidium bromide) was added to the 25µl cell suspension and mixed gently. 10µl of the stained cell suspension was then placed on a clean microscope slide and covered with 22-mm coverslip and analyzed by a confocal microscope. Cells observed under transmitted confocal were washed with 1XPBS and viewed directly under the microscope.

2.18 Determination of Mitochondrial $\Delta\Psi_m$ by flow cytometry:

Potential sensitive probe 3,3' DiOC6 was used to measure mitochondrial $\Delta\Psi_m$. HL-60 cells (1×10^6 cells/ml) were exposed to 50µg/ml of C1, C2, or 100uM of H_2O_2 for 8 hours followed by loading of cells with $DiOC_6$ (40mM for 15 minutes at 37^0C). For positive control, cells were incubated at same time with uncoupling agent CICCP (100µM). Cells were then washed twice with 1xPBS and immediately analyzed in epic profile flow cytometer with excitation set at 488nm. At least 10,000 events were collected per sample and data were analyzed by the WINMDI software.

2.19 Purification of Rat Liver Mitochondria:

Mitochondria were isolated from rat liver (Albino rats, wistar strain). 6-7 rats were sacrificed, and whole liver was dissected on metal tray. After dissection mice were diposed in special waste bags. Rat liver cells were homogenized in 10ml of buffer A and centrifuged at 2250 rpm for 10 minutes at 4^0C. The resulting supernatant (S1) was transferred to another tube and the pellet was resuspended in 10ml of buffer A and centrifuged in the same way. The resulting supernatant (S2) was than mixed with S1 and S1+S2 mixture was centrifuged at 8000rpm for 10 minutes at 4^0C. The resulting pellet was resuspended in 1ml of buffer A and loaded on top of a percoll gradient (prepared in buffer A as 60%, 30%, and 18%) and centrifuged at 8000rpm for 10 minutes at 4^0C. Mitochondria were separated from non-mitochondrial membranes and organelles and collected at 30%/60% interphase. The obtained fraction was washed with 10 volumes of buffer A at 8000rpm for 10 minutes at 4^0C to wash off the percoll. The resulting mitochondrial pellet was resuspended in 1-2 ml of buffer A and kept at 4^0C. All experiment with isolated mitochondria were performed within 4 hours of the preparation. The supernatants derived after treatment with drugs were then analyzed by western blotting.

2.20 Measurement of intracellular pH:

HL-60/CEM cells (1×10^6 cells/ml) were exposed to 50μg/ml of C1, C2, or 100uM of H_2O_2 or pre-incubated with either Catalase (1000U) or RSV (8uM).

Intracellular pH (pHi) was measured by loading cells with the membrane-impermeant BCECF-AM (2',7'-bis(2-carboxyethyl)-5,6-carboxyfluoresce acetoxymethyl ester)

dye (Pervaiz and Clement, 2002). After being subjected to treatment, HL-60 cells were washed with HBSS pH7.4, re-suspended in 100μL HBSS pH 7.4, and loaded with 5μM BCECF-AM and incubated in a 37°C for 30min in the dark. Cells were then washed once with HBSS pH 7.4, re-suspended in 500μL HBSS and analyzed by flow cytometry with excitation wavelength set at 488nm. The fluorescence ratio of 525nm/610nm of at least 10,000 events was used to obtain the pHi from a pH calibration curve. Initially, BCECF-AM enters the cell where its acetate moiety is cleaved by intracellular esterases to BCECF while trapping it intracellularly. BCECF is then able to quench the protons present eliciting a ratiometric fluorescence that is use to determine the pHi.

To generate a pH calibration curve, HL-60 cells without any treatment were loaded with BCECF-AM as above but re-suspended in K^+ buffer with pH ranging from 6.4-8.0 (i.e. 6.4, 6.8, 7.2, 7.6, 8.0). The pH of the K^+ buffer was set using both high and low K^+ solution. Immediately before flow cytometry analysis, these cells were loaded with 20μM of nigericin. Nigericin is a K^+ ionophore that equilibrates the pHi of the cells with the extracellular pH.

2.21 Measurement of intracellular superoxide anion concentration:

HL-60/CEM cells (2×10^6 cells/ml) were exposed to 50μg/ml of C2 or 100uM of H_2O_2 alone or in combination with 8um of RSV.

Intracellular superoxide anion (O_2^-) concentration was assayed by a lucigenin (bis-N-methylacridinium nitrate) based chemiluminescence assay (Pervaiz and Clement, 2002). After being subjected to treatment, HL-60 cells were centrifuged at 4000rpm

for 1min. They were then permeablized in 400µL of 1x somatic cell ATP releasing reagent. Next 100µL freshly prepared lucigenin was autoinjected and chemiluminescence was monitored for 60s (Relative Light Unit/60sec) in the luminometer. Lucigenin is able to react with O_2^- to yield an unstable dioxetane which decomposes, emitting light in the process.

Data are shown as the percentage change in the intracellular O_2^- concentration compared to untreated control cells calculated as:

mean triplicate RLU of treated cells for 60s	x 100%
mean triplicate RLU of control cells for 60s	

2.22 Measurement of intracellular hydrogen peroxide concentration:

HL-60/CEM cells (1×10^6 cells/ml) were exposed to 50µg/ml of C1, C2, or pre-incubated with either Catalase (1000U) or RSV (8uM) depending upon the experiment.

Intracellular concentration of hydrogen peroxide (H_2O_2) was determined by staining the cells with the redox-sensitive CM-H_2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorofluorescin-diacetate) dye (Myhre *et al.*, 2003). After being subjected to treatment, HL-60 cells were washed once with 1x PBS, before being loaded with 5µM CM-H_2DCFDA and incubated in a 37°C incubator for 30min in the dark. The incubated cells were then washed once with its growth medium before being re-suspended in 500µL of it. The cells were analyzed by flow cytometry with excitation wavelength at 488nm and emission wavelength at 525nm. At least 10,000 events were analyzed using the WINMDI software. Initially, CM-H_2DCFDA enters the cell

where its acetate moiety is cleaved by intracellular esterases to C-H_2DCF which trapped it intracellularly. C-H_2DCF is then oxidized by hydrogen peroxide together intracellular peroxidases to yield the fluorescence C-DCF.

2.23 Transient transfection with pcDNA-3 Bax and pIRESRacN17:

Transient transfections of leukemia cells were performed using the SuperFect Transfection Reagents from QIAGEN Gmbh (Germany) as described previously. Briefly, 3μg of the pIRES (empty vector) or pIRESRacN17 /pcDNA-3 Bax in case of HCT116 Bax-/- cells and 2μg of the pCMVβ plasmid encoding for the β-galactosidase protein (β-gal) were added to 20μl of the SuperFect Transfection Reagent. The transfection was carried out as recommended by the vendor. Forty eight hours after transfection, cell lysates were collected and expressions of the transiently expressed RacN17 mutant protein was detected by western blotting using 2μg/ml of a monoclonal anti-human myc epitope antibody or (Boehringer Mannheim, Indianapolis, IN) or2μg ml^{-1} or anti-Bax antibody (BD Pharmigen, San Diego, CA.). Survival of transiently transfected cells was assessed by the β-gal survival assay (Pervaiz et al., 2001).

2.24 β-Gal Survival Assay:

Following triggering of apoptosis with H_2O_2 (100uM) on HCT116 Bax-/- after pcDNA-3 Bax transfection or H_2O_2 (100uM), VCR (1.25ug/ml) on leukemia cells with or without prior addition of RSV(8uM) after RacN17 transfection, β-gal survival assay was performed.

Cell survival was calculated as: [(β-gal activity μg^{-1} protein of transfected cells incubated with the apoptotic trigger)/(β-gal activity μg^{-1} protein of transfected cells incubated without the trigger)] and then converted into percentages. β-gal activity was measured using the Galacto-Star mammalian reporter Kit (TROPIX, Bedford, MA) and measured using the luminometer. Protein concentration was determined using the Commasie Plus protein assay reagent from Pierce (Pierce Chemical Company, Rockford, IL).

3. AIMS

3.1. ***Effect of vitamin C on photo-oxidation of MC540:***

Initially I wanted to investigate the effect of vitamin C on photo-oxidation of merocyanine-540 (MC540) that yields biologically active photoproducts C1 and C2. In addition we wanted to investigate whether addition of vitamin C has any direct effect on photo-degradation of MC540, and as to whether it has any affect on the yield of the isolated compounds.

3.2. ***Involvement of Bax in H_2O_2 induced apoptosis:***

Since we had previously reported that H_2O_2 plays an important role in drug (C2) induced apotosis I wanted to understand the mechanism of H_2O_2 induced apoptosis especially in relationship to Bax, which will perhaps provide some insight on the mechanism of another novel compound C1 derived from the above photo-oxidation model of MC540. I hypothesized that since cell death induced by C2 is H_2O_2 mediated, C1 could function in a similar fashion as mechanism(s) induced by C1 had not been elucidated before. Being derived from the same parent compound it was imperative to study this novel molecule. Since, H_2O_2 could be central to the mechanism of action of the above compounds, the effects of exogenous addition of H_2O_2 on tumor cells would be worth studying.

3.3 ***Redox regulation in tumor cells:***

After establishing the importance of H_2O_2 in drug induced apoptosis I wanted to test our "redox hypothesis" on leukemia cells, to deduce whether a balance between $O_2^{\cdot -}$ and H_2O_2 exists that determines the fate of the cell. To see if this hypothesis holds true I wanted to use anti-cancer drugs (known and unknown) in combination chemotherapy to investigate the intra-cellular redox status. RSV has been shown by our group and many others to have apoptotic effects at higher concentration in the range of 25-200uM depending upon the cell type. I was initially stimulated by a recent report that H_2O_2-induced apoptotic signaling was inhibited in the presence of RSV and the implication that this could be a function of its anti-oxidant activity (Jang and Surh, 2001). Intrigued by these findings I wanted to conduct a series of experiments with low doses of RSV to study if the redox status of the cells can be manipulated and as to whether there is a significant role of this compound as an inhibitor of H_2O_2 mediated drug induced apoptosis. If so, the importance would lie in determining what mechanism(s) are involved in this inhibition with respect to redox regulation.

4. RESULTS

4.1 Vitamin C accentuates photo-oxidation of MC540 to yield biologically active compounds:

4.1.1 Vitamin C upto a dose of 1mM is non toxic to human leukaemia cells:

The apoptosis inducing ability of vitamin C has been extensively highlighted in previous reports (Kang et al., 2003). In our experiments it was imperative to deduce the dose of vitamin C that was non lethal to HL-60 cells and would be the basis of all our further investigation. Results indicate that vitamin C treatment of HL-60 cells for 18hrs, upto a dose of 1mM is non toxic. Henceforth, in our subsequent experiments vitamin C was used at a dose of 1mM unless otherwise stated. **(Fig-6)**

4.1.2 Vitamin C potentiates MC540 killing under photodynamic therapy (PDT) by inducing necrosis:

Leukemia cells (HL-60) were photo-exposed with MC540 or MC540+ vitamin C under white light for 30 minutes and parallel experiments were performed for 30 mins in the dark at 37°C with 20ug/ml of MC540 alone or in combination with 1mM vitamin C. Trypan blue exclusion assay demonstrated that MC540 when activated under light with vitamin C (PDT) exhibited greater cytotoxic potential (75% cell death) as compared with MC540 alone (in light or dark) or in combination with vitamin C.**(Fig-7)**

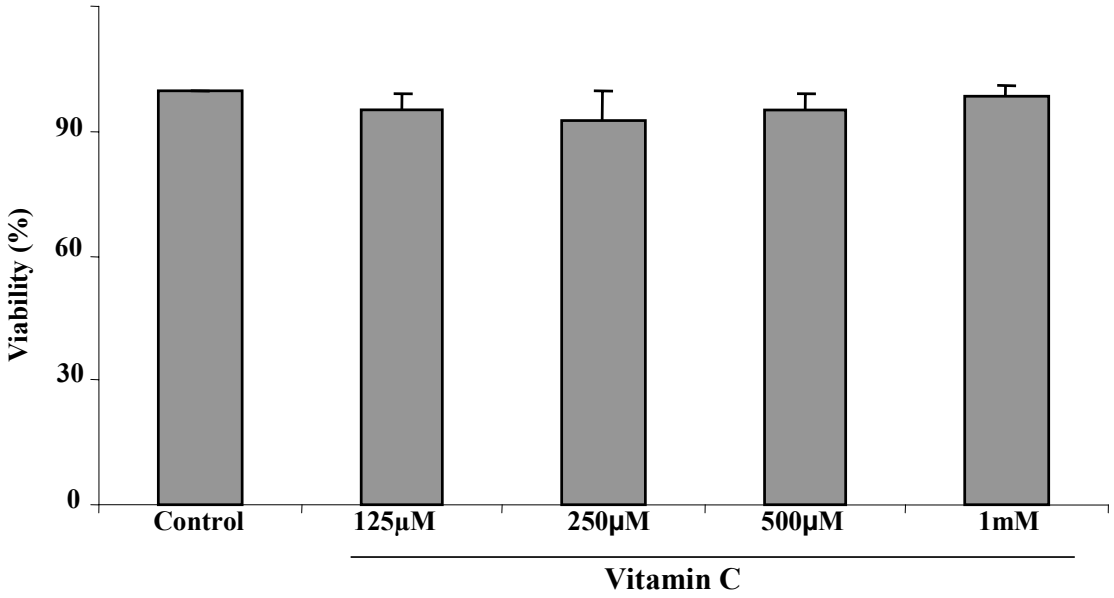

Figure-6: Incubation with Vitamin C (125-1000µM) does not induce cell death in human leukaemia cells.

A total of $1X10^6$ HL-60 cells were treated with increasing concentrations of Vitamin C (µm) overnight. Cell viability was determined by MTT as described in Materials and Methods. Data shown are the mean SD of three independent experiments performed in triplicate.

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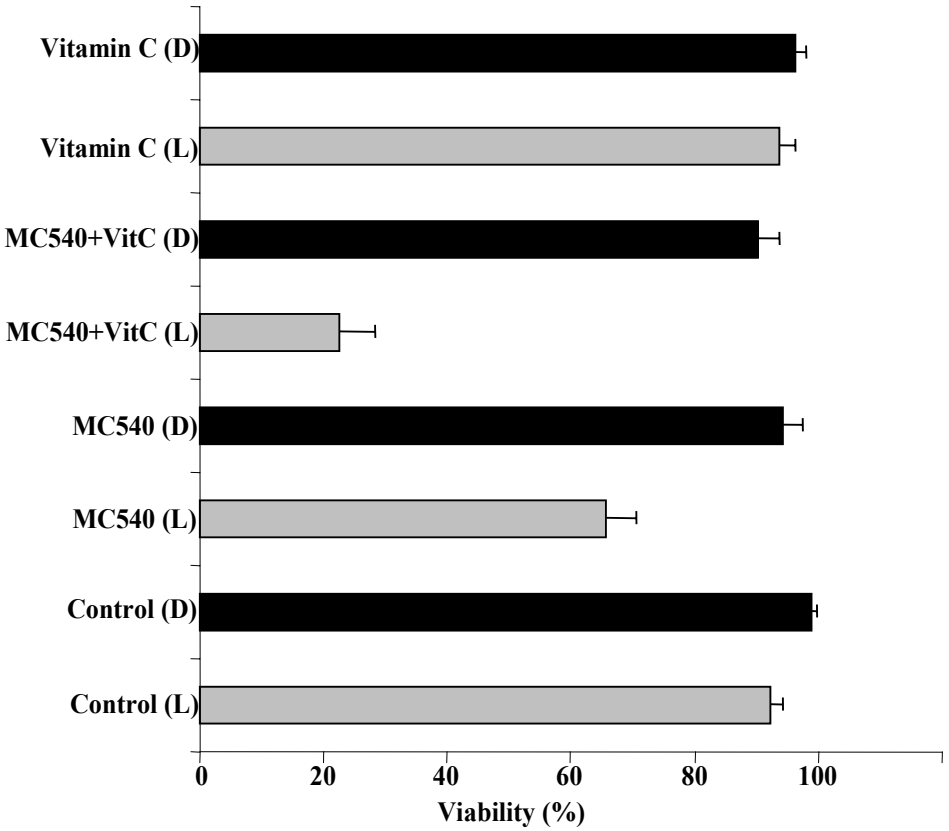


Figure-7: Tumor cell response to PDT is enhanced using MC540 and Vitamin C.

HL-60 cells ($1X10^6$) were incubated with 20ug/ml of MC540 and 1mM Vitamin C, alone or in combination in dark (**D**) denoted by black bars and light (**L**) denoted by grey bars for 30mins. Cytoxicity was assayed using trypan blue exclusion assay as described in Materials and Methods.

To asses the nature of cell death, cells were stained with a mixture of 100ug/ml of acridine orange ethidium bromide and viewed under a confocal microscope. Images clearly revealed the necrotic pattern observed in MC540/MC540+Vit C under PDT. Live HL-60 cells are stained green and necrotic cells were stained orange red indicating loss of membrane integrity. None of the cells showed uniform staining of condensed chromatin, which is suggestive of late apoptotic cells (multiple yellow green dots) and neither there was any increase in detection of sub-G1 fraction of cells by propidium iodide staining. The number of necrotic cells was increased in MC540+Vit C mixture then MC540 alone treatment under light. There was no effect of this mixture in the dark and rest of the light controls appeared normal **(Fig-8)**. These results were re-confirmed by viewing live cells by transmitted confocal microscopy. Indeed did the MC540+Vit C mixture under light shows cells under going tightening of the nuclear membrane, cytoplasmic vacuololization and large nuclei in diameter when compared to control cells. After 30 mins, cells became swollen and died by classical necrosis **(Fig-9)**.

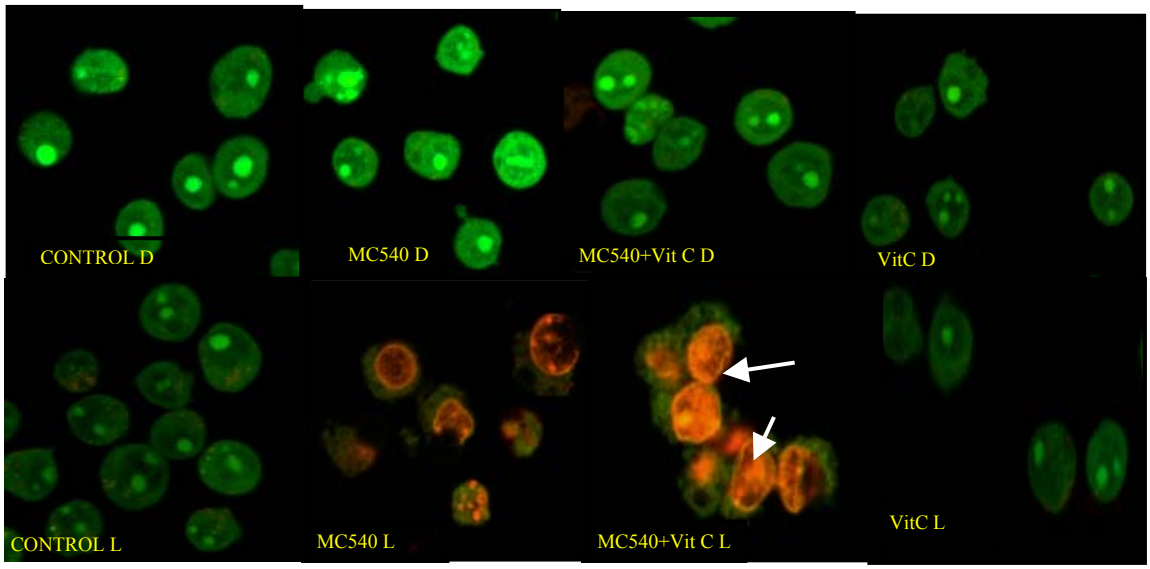


Figure-8: Acridine Orange/Ethidium Bromide staining of MC540 and vitamin C under PDT: necrotic mode of cell death.

HL-60 cells ($1X10^6$) were incubated with 20ug/ml of MC540 and 1mM of vitamin C, alone or in combination in dark (**D**) and upon exposure to light (**L**) for 30mins as shown above.
Cells were stained with 100ug/ml of acridine orange/ethidium bromide and viewed under confocal microscopy.

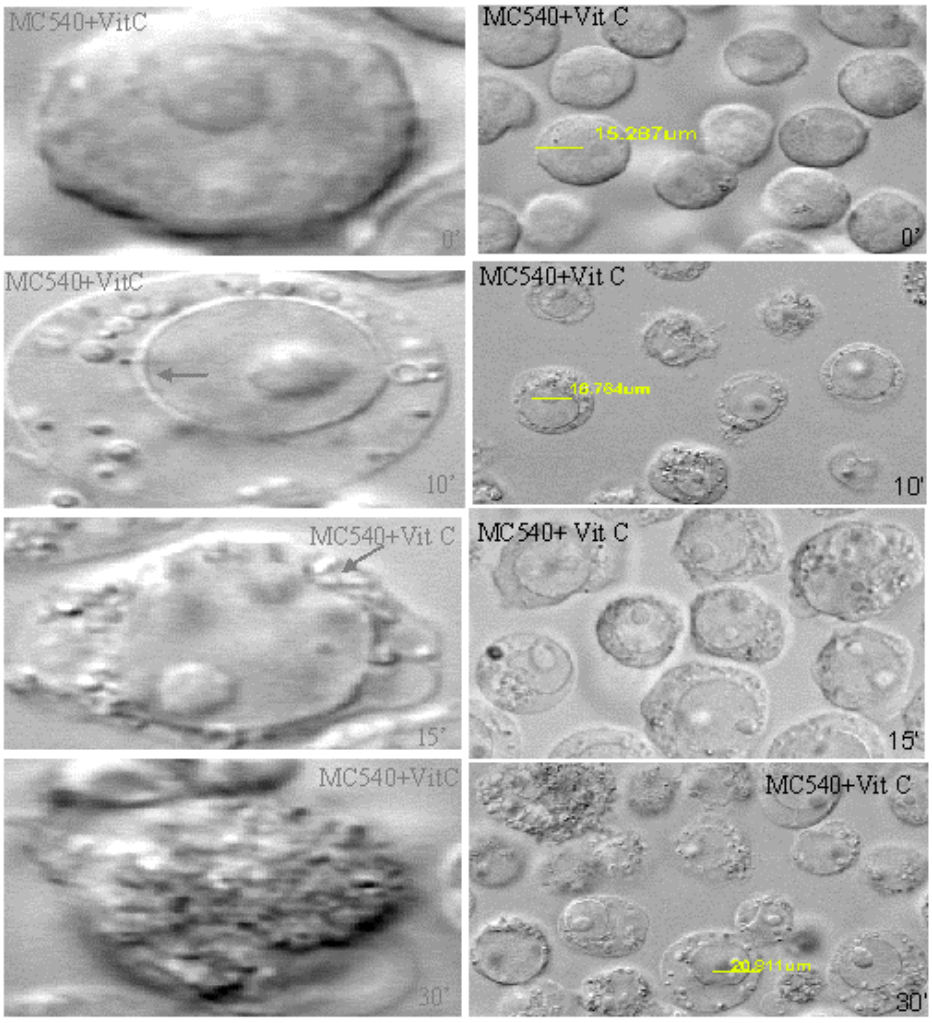


Figure-9: MC540 and Vitamin C under PDT enhances cytotoxicity in leukemia cells: necrotic mode of cell death.

HL-60 cells ($1X10^6$) were incubated with 20ug/ml of MC540 and 1mM of vitamin C. Transmitted confocal images of HL-60 cells were captured in single cell profile (left panel) or whole cells (right panel) were taken at 0, 10, 15 and 30 minutes of exposure.

4.1.3 Complete degradation of vitamin C with pre-activation of MC540 {p(MC540+VitC)} results in increased yield of photo-products with enhanced cytotoxicity towards leukemia cells **:**

MC540 was photo-activated in the presence of vitamin C under white light for 24 hrs. The HPLC system consisted of a Waters 2690 solvent delivery system. Absorption spectrum for vitamin C at 244nm was measured against retention time. The graph clearly showed a decline in vitamin C concentration and by 12 hours almost all of the vitamin C had been degraded as demonstrated by the declining in the absorption peak. There was no detection of absorption for vitamin C at 24 hours **(Fig-10)**. Contrary to my expectation, this suggested that vitamin C was not present in the final photo-activated mixture. The derived mixture was labelled p(MC540+VitC) for simplicity, although as shown above no vitamin C was detected. Thus the final compound was still pMC540 but derived by photo-oxidation with vitamin C. Small (p) denotes pre-activation.

After freeze drying, samples were suspended in 100% DMSO. Spectral analysis of pMC540 showed a complete shift of the absorption maximum from 568 to 280nm suggesting its complete photo-degradation (photo-bleaching) of the parent compound. This absorption maximum was accelerated in the presence of vitamin C at 6 hours compared to the peak of MC540 alone. Furthermore, the yield is enhanced in p(MC540+VitC) (1.5A) then pMC540 (0.75A) alone **(Fig-11)**. Table-3 summarizes the findings on yield of the photo-products.

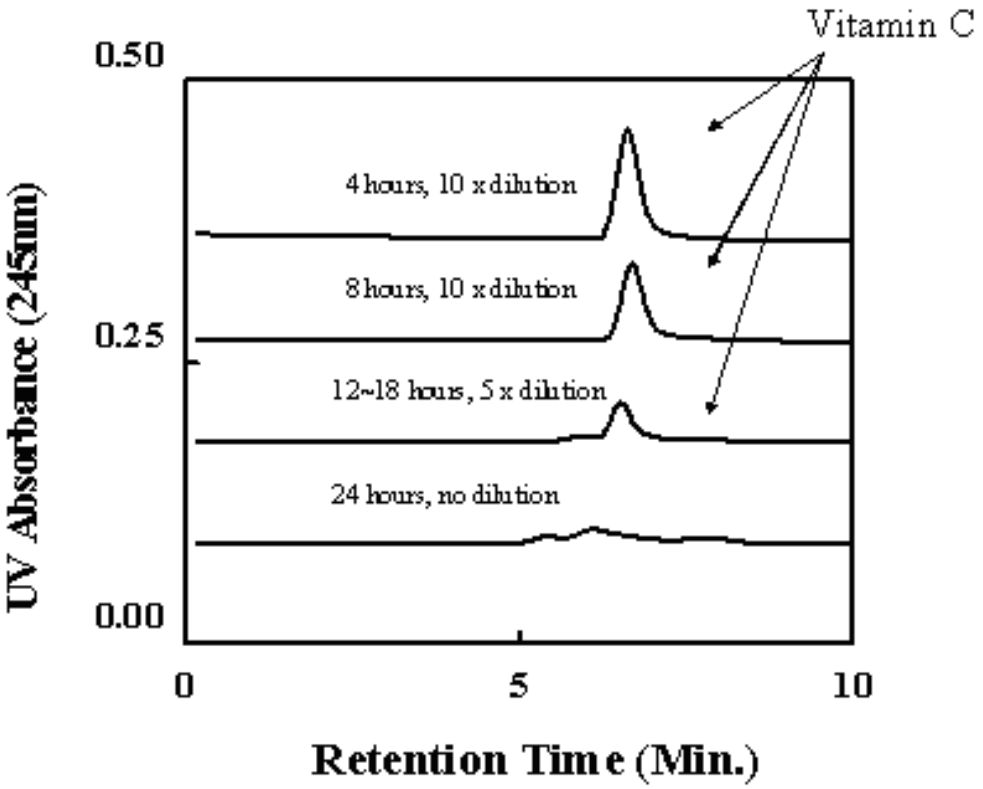


Figure-10: Vitamin C added is completely degraded during photo-activation of MC540.

Concentration of Vitamin C was measured 4-24 hours by HPLC (Waters, Inc.) using the absorption maximum of 245nm over retention time (0-10mins) in the compound p(MC540+VitC).

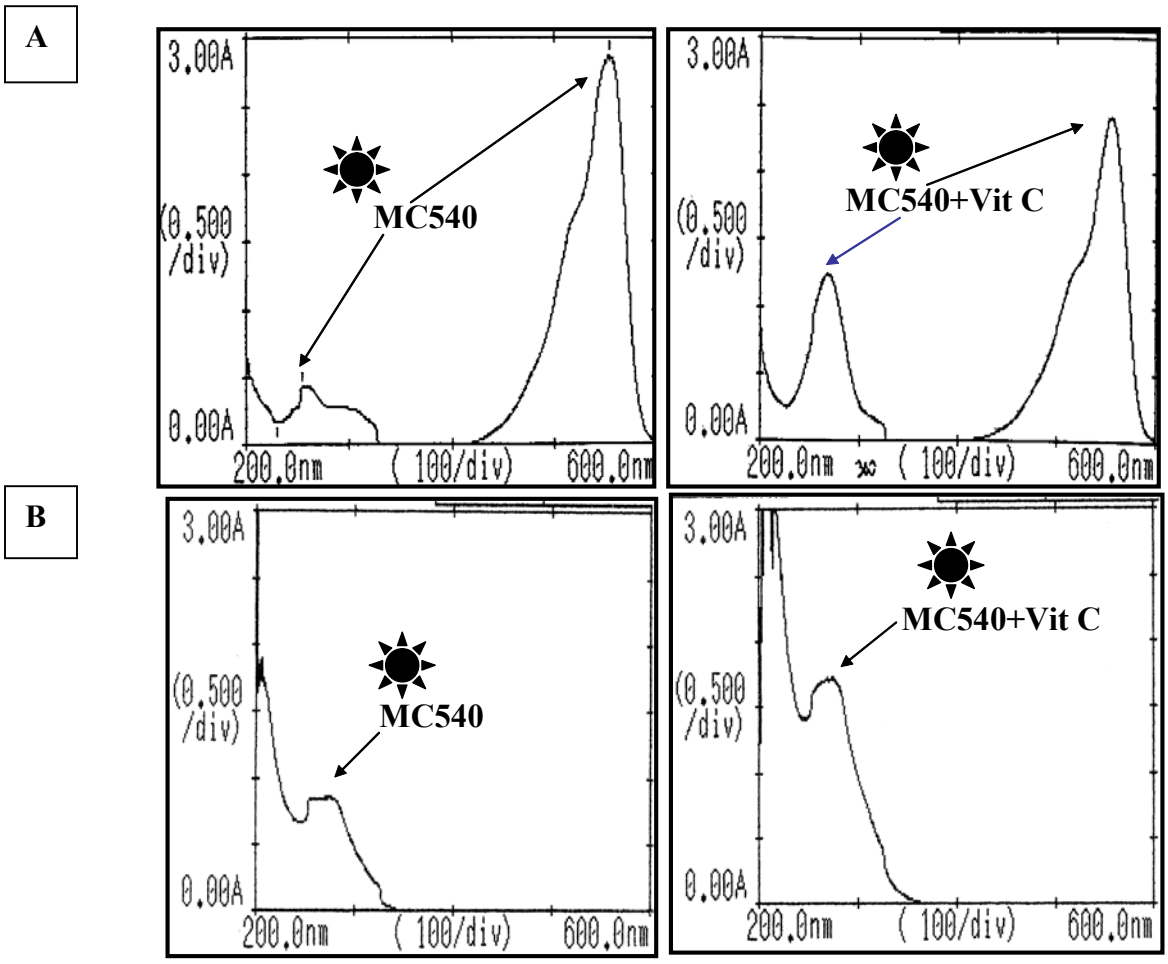


Figure-11: Photo-activation of MC540 is accelerated by Vitamin C.

Spectrophoto-metric analysis from 190nm-600nm against Armstrong (A) of photo-activated merocyanine alone *MC540 and in the presence of vitamin C (*MC540+VitC) at **(A)** 6 hours and **(B)** 24 hours. The graphs were directly obtaine using spectro-photometer from SLT Lab Systems (Austria). After 24 hours of photo-activation the samples are labelled as pMC540 and p(MC540+VitC) respectively.

Table 3-Production of photo-product(s) of pMC540 and p(MC540+VitC):

Drugs	pMC540 / mg	p(MC540 + vit C) / mg
C1	1 ± 0.5	1.5 ±0.75
C2	1.75 ±0.75	9.5 ±0.5

Comparison of cytotoxicity on HL-60 cells between pMC540 and p(MC540+VitC) stored at -30° C on HL-60 cells over 1-60 days reveal that p(MC540+Vit C) mixture exhibits greater cytotoxic potential at 40% when compared to 70% with pMC540. Although pMC540 lost its cytotoxic potential after 60 days, p(MC540+VitC) maintained its toxicity to 40% indicating better stability of the newly derived compound **(Fig-12A)**.

4.1.4 p(MC540+VitC) apoptotic signaling in leukemia cells:

In contrast to (light+MC540+VitC+ tumor cells) under photodynamic therapy, the newly activated compound p(MC540+VitC) demonstrated increased capase 3 activity suggestive of apoptotic potential than the combination used in PDT. **(Fig-12B)** Cytotoxic potential of the drug can be noted by MTT assay demonstrating that potentiation was only a result of vitamin C added together and not of vitamin C added from outside. PARP, an enzyme involved in DNA repair is proteolytically cleaved from 116 to 89kDa and is a classical hallmark of apoptosis. The cleavage was significantly greater in HL-60 cells treated with p(MC540+VitC) overnight then with other samples (see descriptive figure legend **(Fig-13)** Using MC540 together with photo-activated vitamin C added separately from outside or addition of unexposed vitamin C had no effect on the cytoxicity induced by MC540 on leukemia cells. The

main advantage of deriving the new compound after pre-activation was by passing the use of light and in addition p(MC540+VitC) now induces apoptosis rather than necrosis. Now the drug could potentially be used as an apoptosis inducing agent.

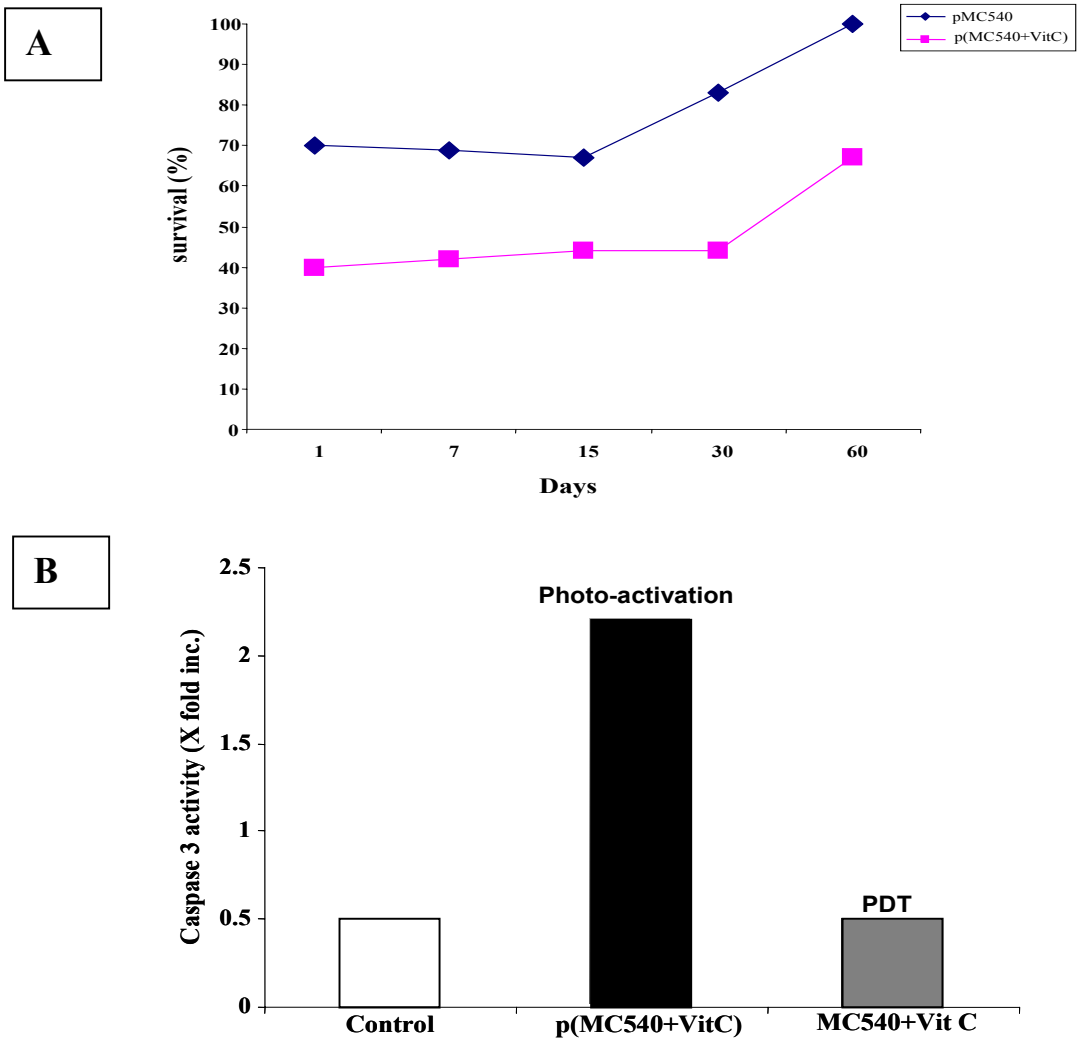


Figure-12: p(MC540+VitC) sustains it cytotoxic potential in comparison to pMC540.

(A) $1X10^6$ HL-60 cells were assayed by MTT over time following incubation with 100ug/ml of pMC540 or p(MC540+VitC) for 18 hours stored at -80 ^oC for 1, 7, 15 , 30 and 60 days.

(B) Caspase 3 activity measured in HL-60 cells ($1X10^6$) treated with MC540 (20ug/ml) +Vit C (1mM) under PDT and pre-activated mc540+Vit C for 12 hours.. Final concentration used for p(MC540+Vit C) was 100ug/ml.

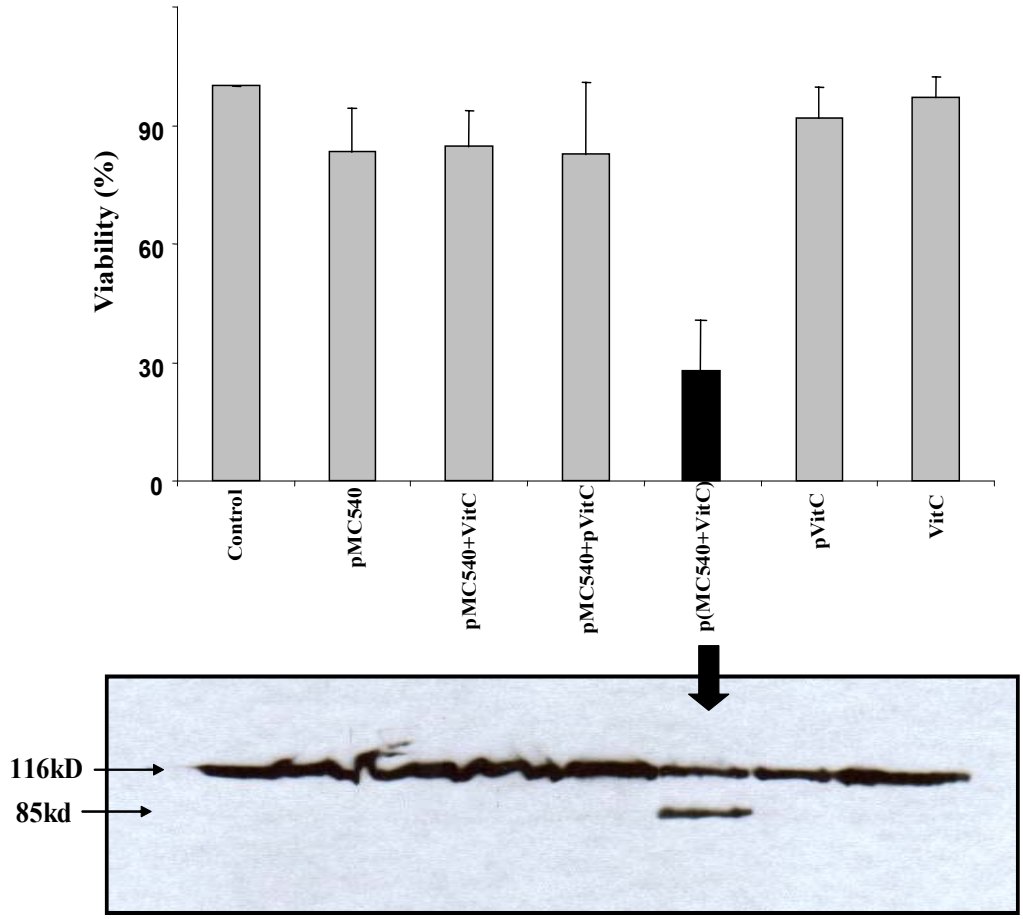


Figure-13: p(MC540+VitC) has greater apoptosis inducing ability then its counterparts.

pMC540540 100ug/ml of pre-activated MC540 alone.
pMC540+Vit C- 100ug/ml of pre-activated MC540 with 1mM of VitC added from outside.
pMC540+pVit C- 100ug/ml of pre-activated MC540 with 100ug/ml of photo-activated Vit C.
p(MC540+VitC)- 100ug/ml of preactivated merocyanine in the presence of Vit C.
pVit C- 100ug/ml of photo-activated Vit C alone.
Vit C- 1mM of Vit C alone.

Cleavage of the caspase 3 substrate PARP was assessed by western blotting using a monoclonal anti-PARP (clone C2-10) antibody in lysates of $2x10^6$ cells following exposure to corresponding samples for 18 hours. The 85kD band indicates caspase 3 mediated apoptotic cleavage of PARP.

4.1.5 The mechanism of apoptosis induced by p(MC540+Vit C) involves intra-cellular generation of hydrogen peroxide:

The enhanced apoptosis induced by p(MC540+VitC) was indeed dependent on intra-cellular H_2O_2 production. The significant rightward shift observed in p(MC540+VitC) could be blocked by using H_2O_2 scavenger catalase **(Fig-14)** consequently leading to block in cell death **(Fig-15)**. Apoptosis induced by p(MC540+VitC) was significantly blocked by pre-incubating leukemia cells with scavengers of H_2O_2 (Catalase, Euk-8 and Mannitol) **(Fig-16)**.

Photo-products of MC540, merodantoin (C1) and merocil (C2) are novel molecules currently being investigated in our laboratory for their biological activity (Pervaiz et al., 1999b).

4.1.6 Photoproduct(s) of MC540 (C1 and C2) induce mitochondrial apoptosis:

Both photo-product(s) C1 and C2 derived from photo-oxidation of MC540 are classical apoptosis inducing agents. Data demonstrated here is on C2 and used as a representative experiment. Similar results were obtained for C1. Forward scatter was significantly decreased using C2 indicatiing a decrease in cell size **(Fig-17A)**. The cells were shrunken in diameter on transmitted confocal micrscopy **(Fig-17B)** and acridine orange/ethidium bromide staining showed yellowish apoptic bodies or clusters in comparison to live cells that stained green **(Fig-17C)**. This was in sharp contrast to MC540+ VitC in PDT that demonstrated necrosis.

The two photo-product(s) engage the mitochondria by a drop in transmembrane potential **(Fig-18)**, resulting in Bax translocation to the mitochondria and eventually

leading to cytochrome C release **(Fig-19)**. Data shown here is on C2 as a representative experiment. Mitochondrial apoptosis inducing ability on C1 is shown and discussed later in this thesis.

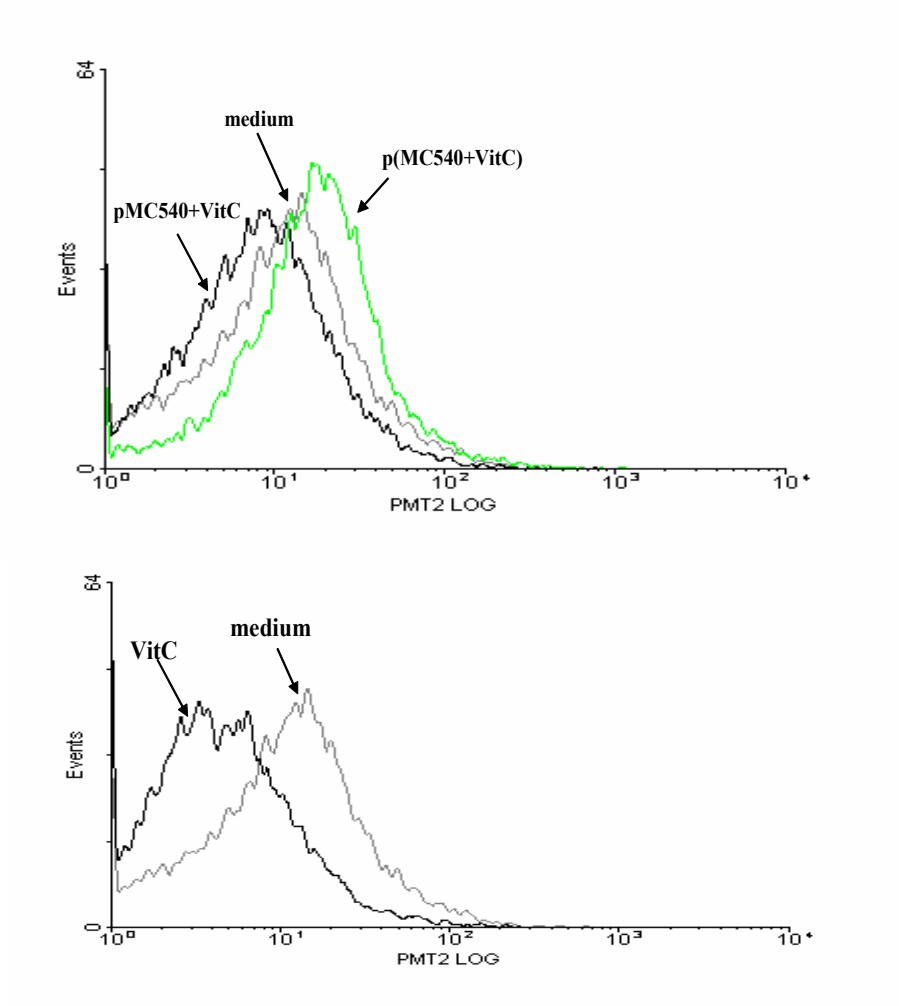


Figure-14: p(MC540+Vit C) stimulates intra-cellular production of H_2O_2.

HL60($1x10^6$) cells were treated with 100ug/ml of p(MC540+VitC) and 1mM of Vit.C alone or added with 100µg/ml pMC540 for 6 hours**.** Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5µM) for 30 minutes and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry, as described in Materials and Methods.

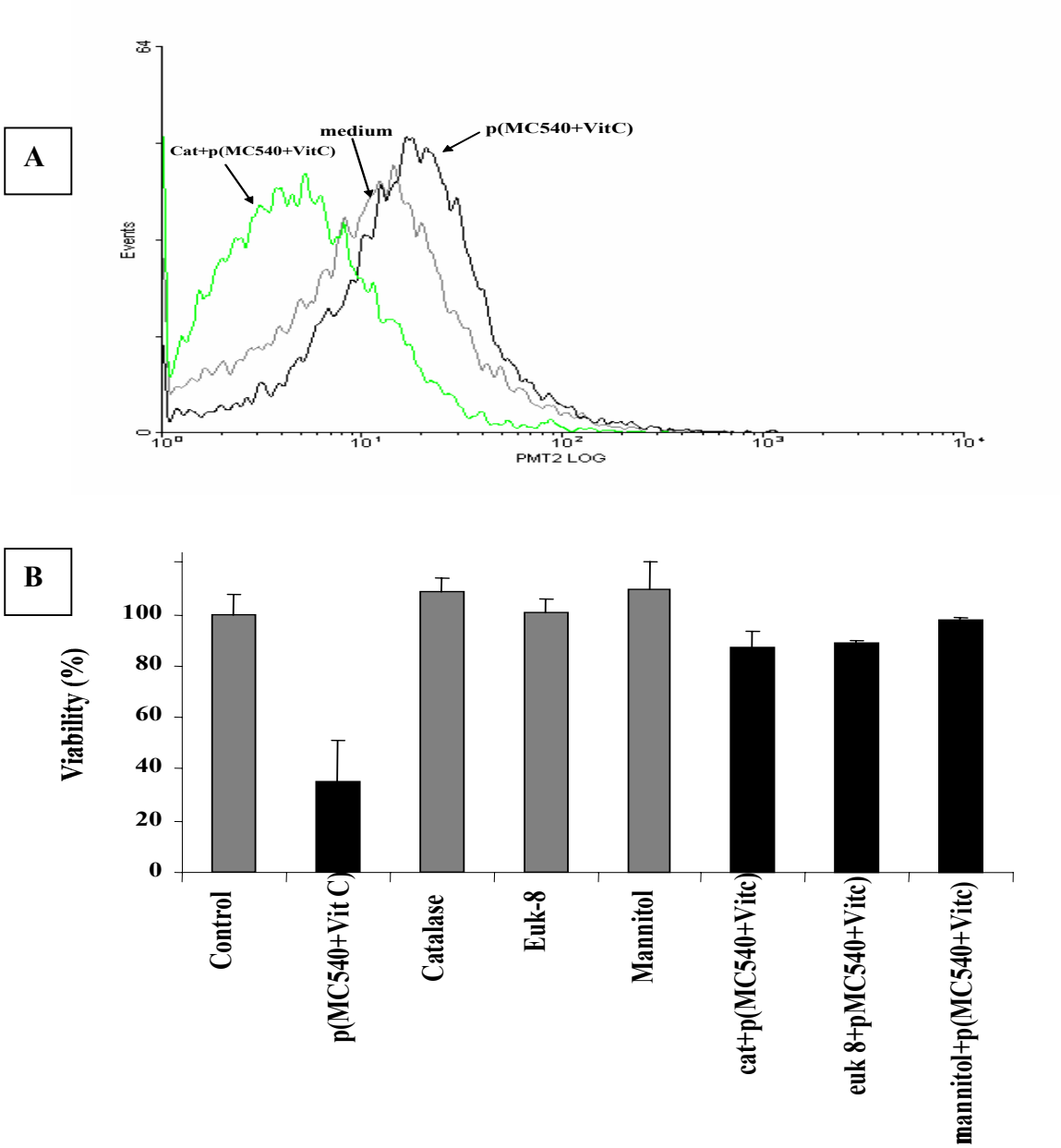


Figure-15: Catalase inhibits hydrogen peroxide production and cell death triggered by p(MC540+Vit C).

(A) HL60 ($1x10^6$) cells were treated with 100µg/ml of p(MC540+VitC) for 6 hours in the presence or absence of 1000U/ml of catalase**.** Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5µM) for 30 min. and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry, as described in Materials and Methods.

(B) $1x10^6$ cell/ml were incubated with 100µg/ml of p(MC540+VitC) for 18 hours or prior to the addition of 1000U/ml of catalase, 20µM of Euk-8, and 1uM mannitol. Cell viability was determined by the MTT assay as described in Materials and Methods. Data are shown as Mean S.D. of at least three independent experiments performed in triplicates.

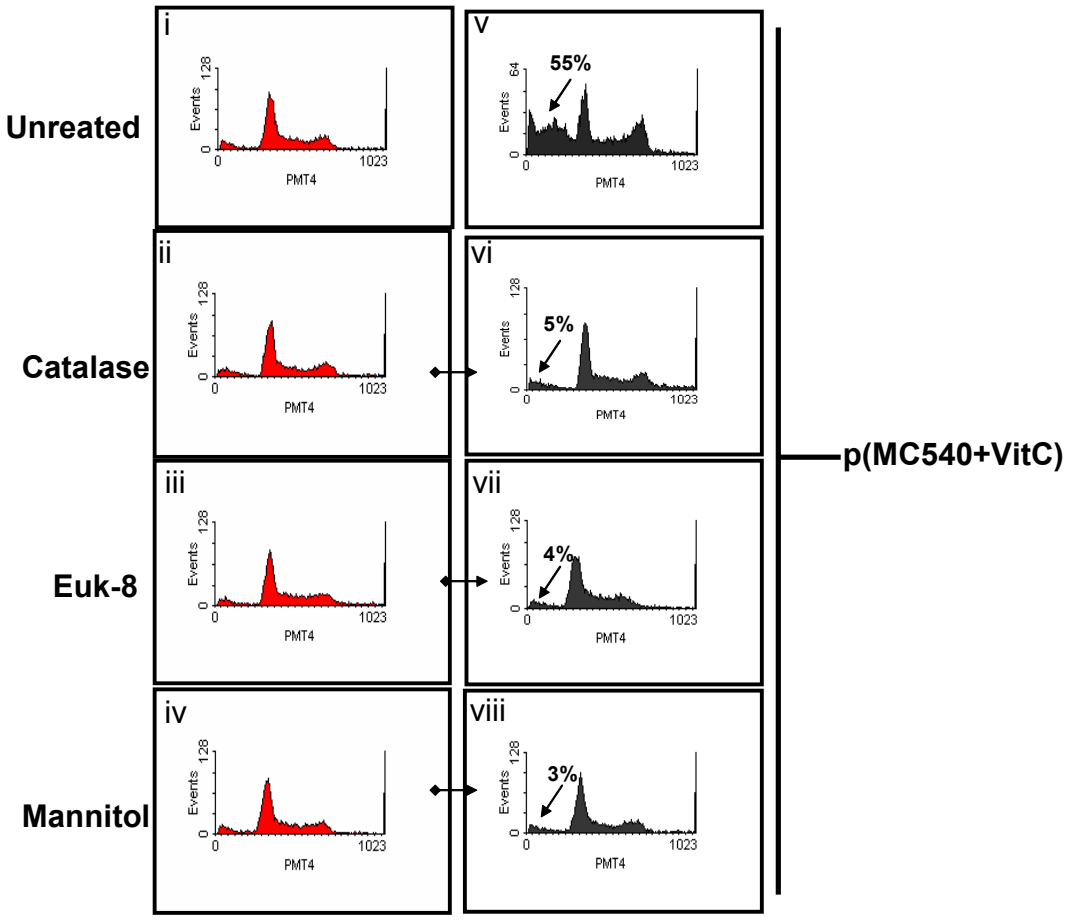


Figure-16: Catalase inhibits apoptosis triggered by p(MC540+Vit C).

DNA fragmentation was determined by PI staining and the appearance of sub-G1 fraction. $1x10^6$ cells were treated as above, permeablized and stained with PI as described in Materials and Methods. Cells were analyzed by flow cytometry (10,000 events) and numbers denote percentage of cells in sub-G1 are shown at various phases of the cell cycle. Panels i, ii, iii, iv are untreated, catalase, euk-8, mannitol respectively, panel v is p(MC540+VitC) alone while panels vi, vii, viii are pre-incubated with catalase, Euk-8, mannitol for 1 hour with subsequent addition of p(MC540+VitC). The number denotes the percentage of apoptotic cells in sub-G1. Doses used were the same as in Figure-15.

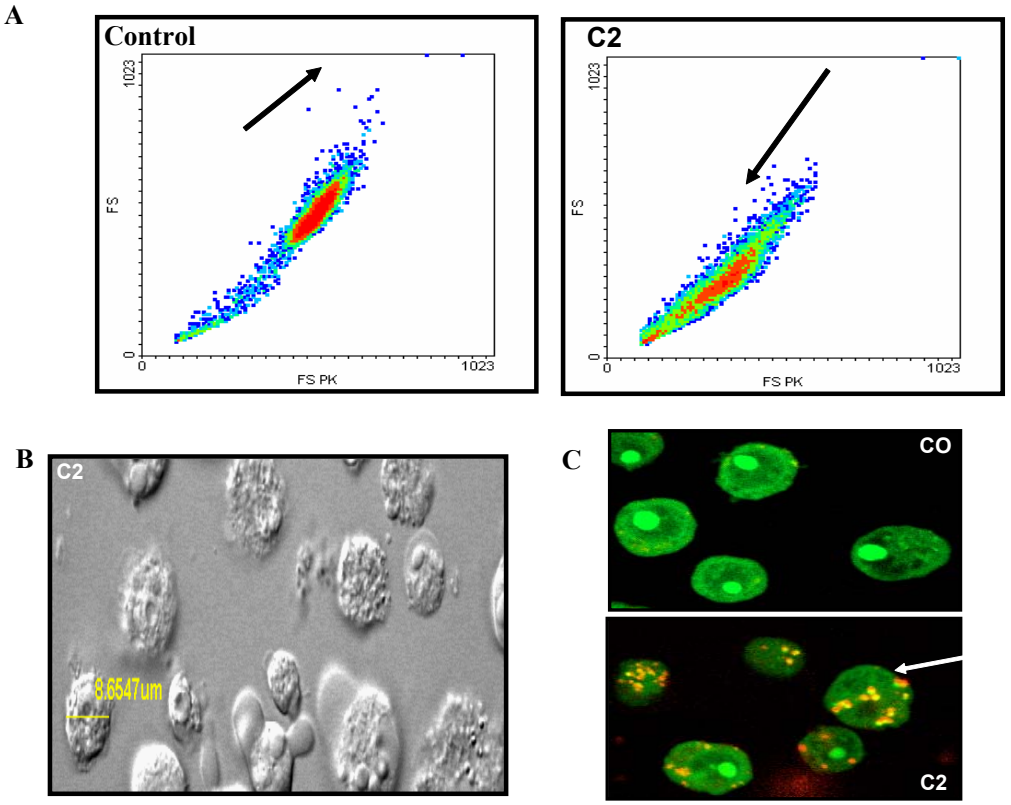


Figure-17: Novel compounds C1 and C2 induce apoptosis in leukemia cells. Apoptosis profile of C2 is shown as a representative experiment.

(A) $1x10^6$ cells were treated with C2 and cell size was determined using flow cytometry as decribed in Materials and Methods.

(B) HL-60 cells ($1X10^6$) were incubated with 50ug/ml of C2 and transmitted confocal images were obtained using Zeiss LSM confocal microscope.

(C) HL-60 cells ($1X10^6$) were incubated with 50ug/ml of C2 and dually stained with 100ug/ml of acridine orange/ethidium bromide for 1 min and viewed under confocal.This was to assess the formation of apoptotic bodies.
Similar results were obtained for C1.

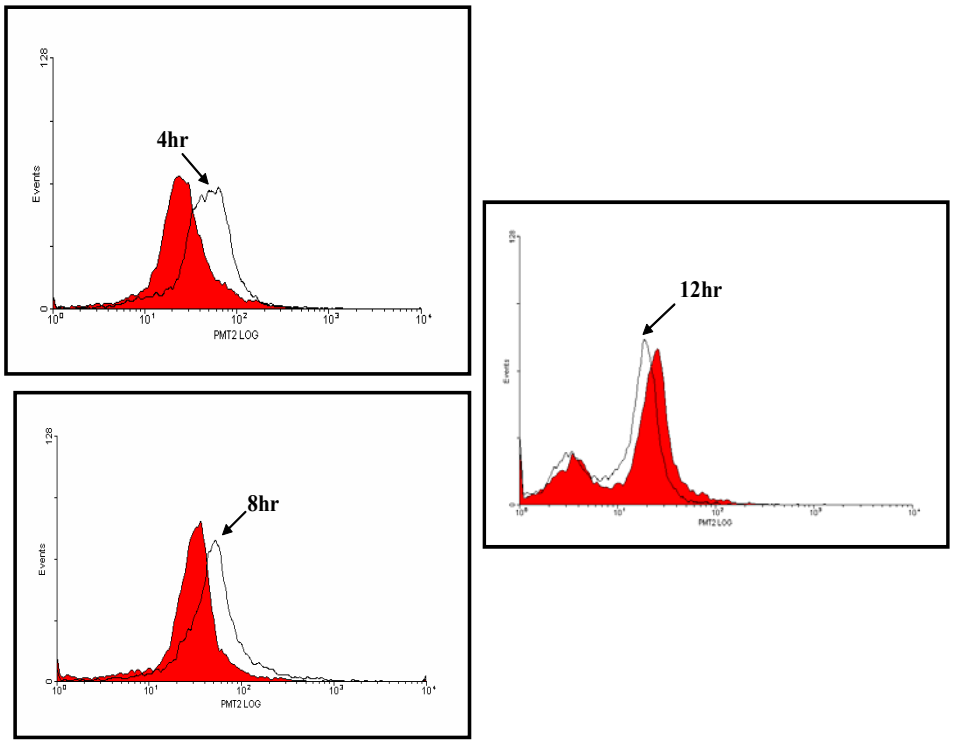


Figure-18: C1 and C2 drops mitochondrial transmembrane potential in HL-60 cells prior to the release of cytochrome C

HL60 cells ($1x10^6$/ml) were incubated with C2 or C1 (50μg/ml for 6hrs) and the change in mitochondrial membrane potential ($\Delta\psi_m$) was assessed by flow cytometry using $DiOC_6$ as described in Materials and Methods. Data shown here is on C2. C1 drops transmembrane potential in a similar fashion.

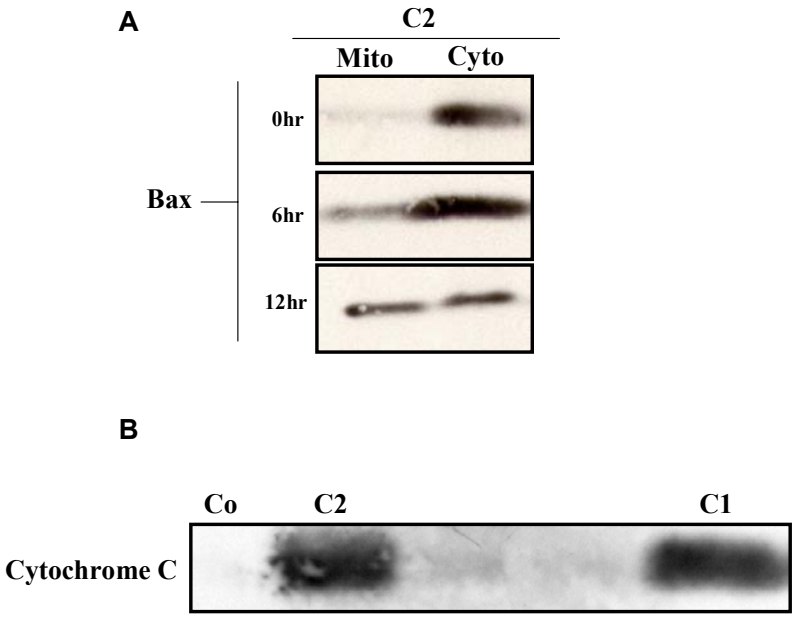


Figure-19: C1 and C2 engage the mitochondria by Bax translocation and subsequent release of cytochrome C.

HL-60 cells were incubated with C2 and cytosolic fractions were purified as described in Materials and Methods. For CytC, mitochondria were purified from rat liver and supernatants subjected to treatment with C2 and C1. Western blots were probed with anti-Bax and anti-CytC in as described in Materials and Methods. Western blot analysis is shown on C2. Bax translocation by C1 is shown later in the thesis.

4.1.7 Preface to next series of experiments

Thus, we identified that H_2O_2 as a mediator of reactions induced by compound(s) C1 and C2 derived from photo-oxidation model of MC540.

The exact mechanism of action is unclear of this novel bio-active molecules but since H_2O_2 is strongly implicated in reactions induced by these compounds on tumor cells I wanted to ask the question whether generation of H_2O_2 is a critical effector mechanism in drug induced apoptosis and perhaps questioning the mechanism of H_2O_2 mediated siganling. Henceforth, tweaking the redox status or altering environment by H_2O_2 production that favours apoptosis in tumor cells and simultaneously discovering novel compounds that exploit this system would lead to the generation of effective chemotherapeutic agents. Depending upon its intracellular concentration, H_2O_2 has been shown to play a role in processes as diverse as proliferation and cell death(Burdon, 1995; Clement and Pervaiz, 2001; Hampton and Orrenius, 1997).

However, its effect directly involving the Bcl-2 family of proteins remains clouded. Here I investigate the crucial involvement of pro-apoptotic protein Bax in H_2O_2 induced death signaling. Moreover, any data generated on C1 a compound derived from photo-oxidation from MC540 would provide important information on its mechanism of action that has not been previously reported. Henceforth, in this thesis I have used two model drugs C1 and C2 in two different scenarios to investigate H_2O_2 mediated apoptotic signaling and to understand the redox regulation in tumor cell survival or cell death.

4.2 Involvement of H_2O_2 in drug induced Bax activation in tumor cells

4.2.1 Critical involvement of Bax in response to exogenous H_2O_2 :

The functional importance of Bax during death execution has been linked to its translocation from the cytosol to the mitochondria, therefore we were interested to investigate if the cellular distribution of Bax was affected by H_2O_2 exposure. HL60 and CEM cells underwent classical apoptosis upon exposure to 100μM of H_2O_2 as shown by the increase in the activities of caspases 9 and 3 **(Fig-20A)** and DNA fragmentation denoted by appearance of sub-G1 population on P/I staining that could be inhibited by catalase **(Fig-20B, 21)**. In addition, exposure of HL60 cells to exogenous H_2O_2 resulted in a significant depolarization of the mitochondrial transmembrane potential and cytosolic release of Cyt. C **(Fig-22A, C).** Interestingly, the drop in transmembrane potential and release of cytC was preceded by cytosolic Bax being redistributed to the mitochondrial fraction in HL60 cells **(Fig-22B**), which could be inhibited by the H_2O_2 scavenger catalase, but not by the general caspase inhibitor zvad-fmk. The translocation of Bax was observed as the shifting of the cytosolic band to the mitochondria. Membranes were probed with anti-MnSOD antibody (as MnSOD is specifically present in the mitochondria) to provide evidence that the mitochondrial preparation was relatively clean. H_2O_2-dependent Bax translocation inhibitable by catalase was not exclusive to HL-60 cells but was also observed in CEM cells **(Fig-22D)**. In addition, orange yellowish Bax clusters or co-localization of Bax to the mitochondria was observed on confocal microscopy where green represents immuno-staining for Bax and red stands for mito-tracker staining of

the mitochondria **(Fig-23)**. These data clearly provide evidence that H_2O_2 is an effector mechanism for mitochondrial localization of Bax and this effect is not exclusive to one cell type.

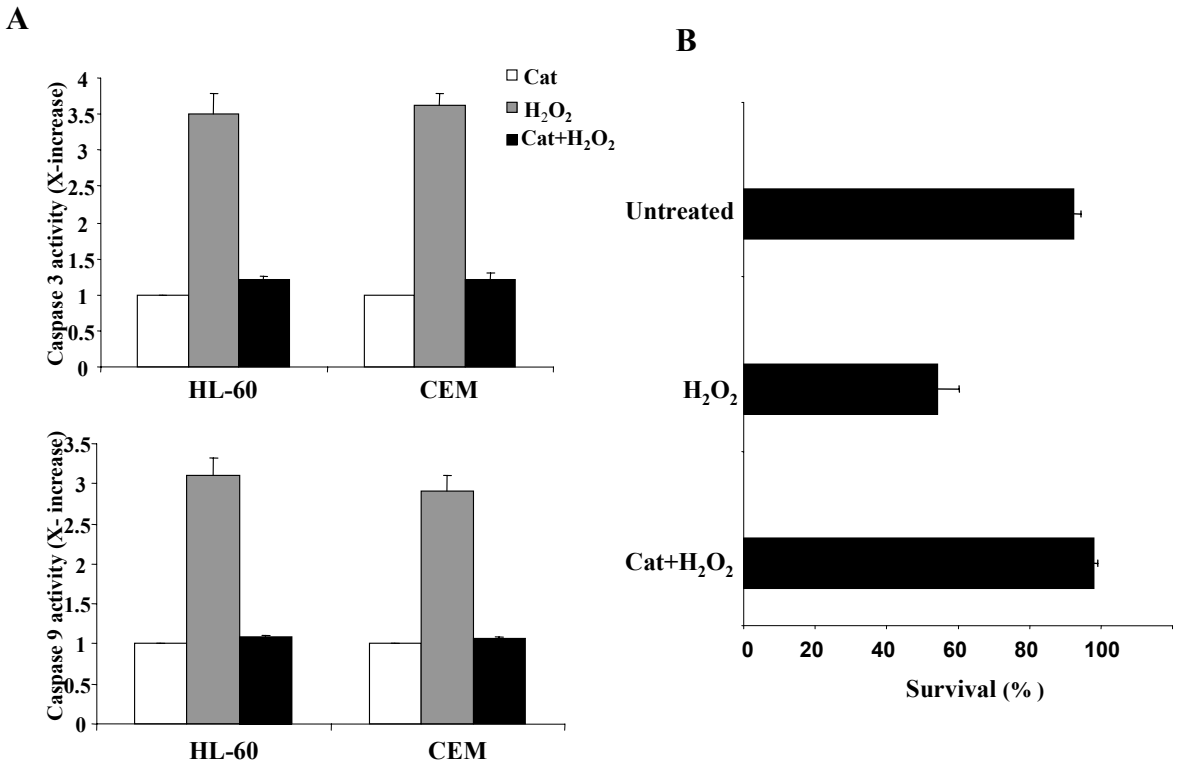


Figure-20: H_2O_2 induced cell death is caspase driven in human leukemia cells.

(A) HL60 and CEM leukemia cells ($1x10^6$/ml) were incubated with 100μM H_2O_2 for 12 hrs in the presence and absence of 1000U/ml of catalase and activities of caspases 3 and 9 were determined as described in Materials and Methods. Data are shown as fold increae (X increase) in activity over the untreated cells (1X) and are Mean±S.D. of three independent experiments.

(B) $1x10^6$/ml) were incubated with 100μM H_2O_2 for 18 hrs in the presence and absence of 1000U/ml of catalase Cell survival was assessed by the MTT assay and the Mean±S.D. of three independent experiments are shown. Statistical analysis of data was performed using the paired *t*-test.

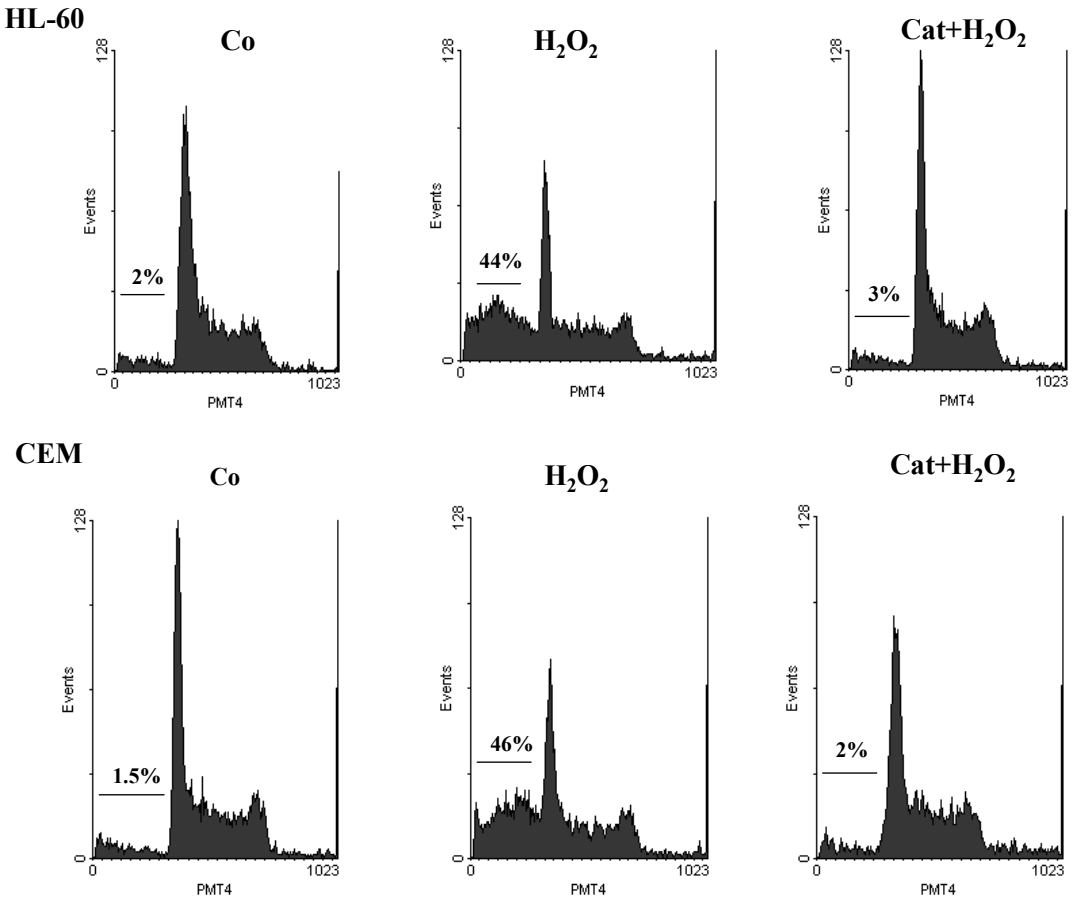


Figure-21: H_2O_2 induced DNA fragmentation is completely blocked by catalase in human leukemia cells.

DNA fragmentation was determined by PI staining and the appearance of sub-G1 fraction. $1x10^6$ cells were treated as in B, permeablized and stained with PI as described in Materials and Methods. Cells were analyzed by flow cytometry (10,000 events) and percentage shown are apoptotic cells in sub-G1.

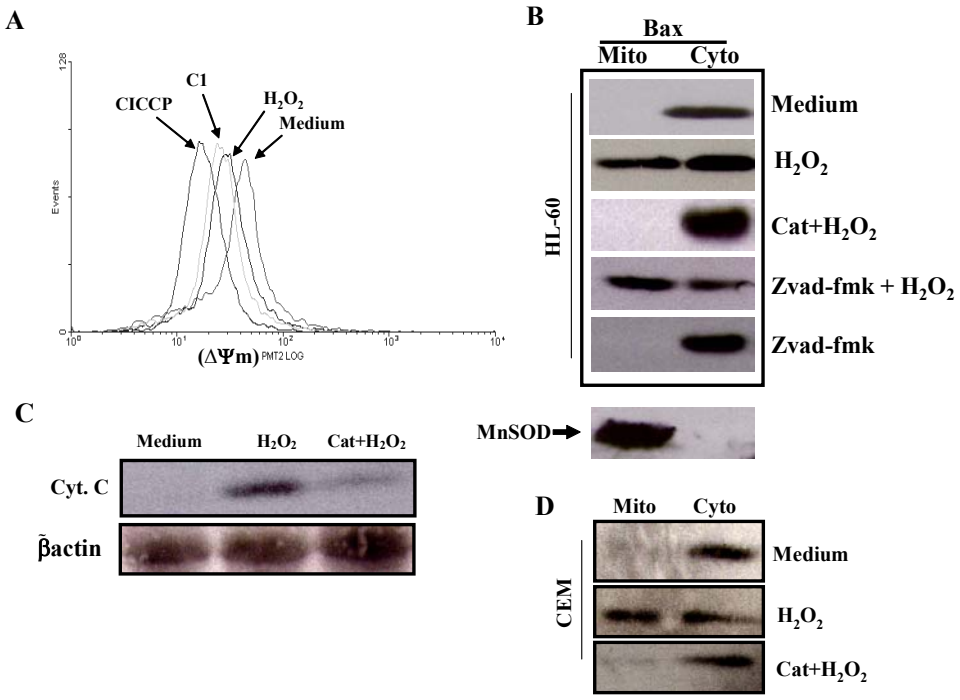


Figure-22: H_2O_2 triggers Bax translocation in human leukemia cells.

(A) HL60 cells ($1x10^6$/ml) were incubated with H_2O_2 (100μM for 6hrs) or C1 (50μg/ml for 6hrs) or CICCP (100μM for 1 hr) and the change in mitochondrial membrane potential ($\Delta\psi_m$) was assessed by flow cytometry using $DiOC_6$ as described in Materials and Methods.

(B) Cytoslic fractions from HL60 cells ($2.5x10^7$) treated with 100μM H_2O_2 for 12 hrs in the presence or absence of 1000U/ml of catalase were subjected to western blot analysis for Cyt. C. Anti-β actin was used as a loading control.

(C) Mitochondrial and cytosolic fractions from HL60 cells ($2.5x10^7$) incubated with 100μM H_2O_2 for 12 hrs in the presence or absence of catalase or ZVAD-fmk were subjected to western blot analysis using anti-Bax. Purity of mitochondrial fraction was confirmed by probing with anti-MnSOD.

(D) Western blot analysis using anti-Bax was also performed on fractions obtained from CEM cells following exposure to 100μM H_2O_2 with or without catalase.

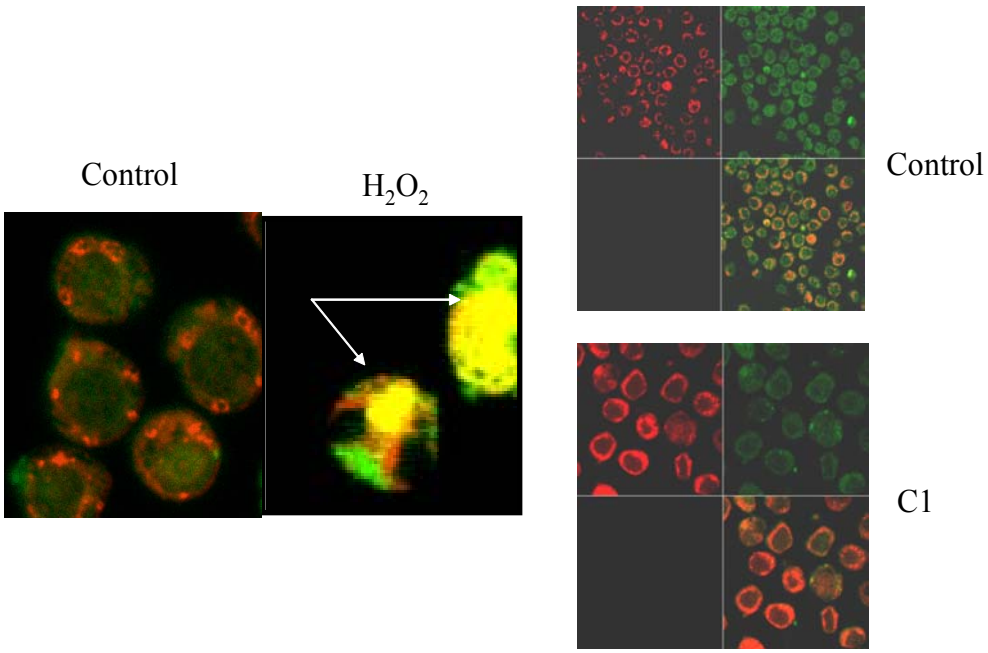


Figure-23: H_2O_2 and C1 form Bax clusters by co-localization to the mitochondria

$5x10^6$ HL-60 cells were stained using Bax-monoclonal anti-body (1:100) with secondary FITC (1:50) and mitotracker (red) after treatment with H_2O_2 (100uM) or C1 (50ug/ml) as described in Materials and Methods. Cells were mounted on cover slips and after immuno-staining were directly viewed under confocal microscope. (LSM-ZEISS at NUMI, CRC, NUS).

4.2.2 Drug-induced Bax translocation during apoptosis of cancer cells is H_2O_2 mediated:

Having demonstrated that exposure of cells to exogenously added H_2O_2 resulted in translocation of Bax in human leukemia cells, I next asked if this was also the mechanism utilized by anti-cancer drugs that trigger apoptosis by inducing intracellular production of H_2O_2. In order to do so, HL-60 cells were exposed to a novel photo-product of MC540 C1 (merodantoin), the apoptosis inducing activity of which has been previously reported by the group (Pervaiz et al., 1999b). Exposure of HL60 cells to 50μg/ml of C1 for 6 hours resulted in a dramatic increase in the intracellular production of H_2O_2 **(Fig-24A)**. In addition, exposure to C1 resulted in a significant decrease in cell survival **(Fig-24B)**, increase in caspase 9 activity and cleavage of PARP (**(Fig-24C, Fig-24D)**. Furthermore, western blot analysis of cytosolic and mitochondrial extracts prepared from HL-60 cells treated with C1 (50ug/ml for 12 hours) showed translocation of Cyt. C from mitochondria to the cytosol **(Fig-24E)**, and the reciprocal translocation of Bax from the cytosol to the mitochondrial fraction, similar to that observed for exogenous H_2O_2 **(Fig-24F)**. Bax co-localization with C1 treatment was also observed on confocal imaging as shown previously in Fig-23. These changes could be inhibited by 1000U/ml of catalase as shown in the individual results in **(Fig-24A-F)**, thus strongly implicating H_2O_2 as the mediator of mitochondrial recruitment and apoptosis triggered in tumor cells by C1. The results were substantiated by staining for DNA fragmentation induced by C1 that could be significantly blocked by catalase **(Fig-25)**.

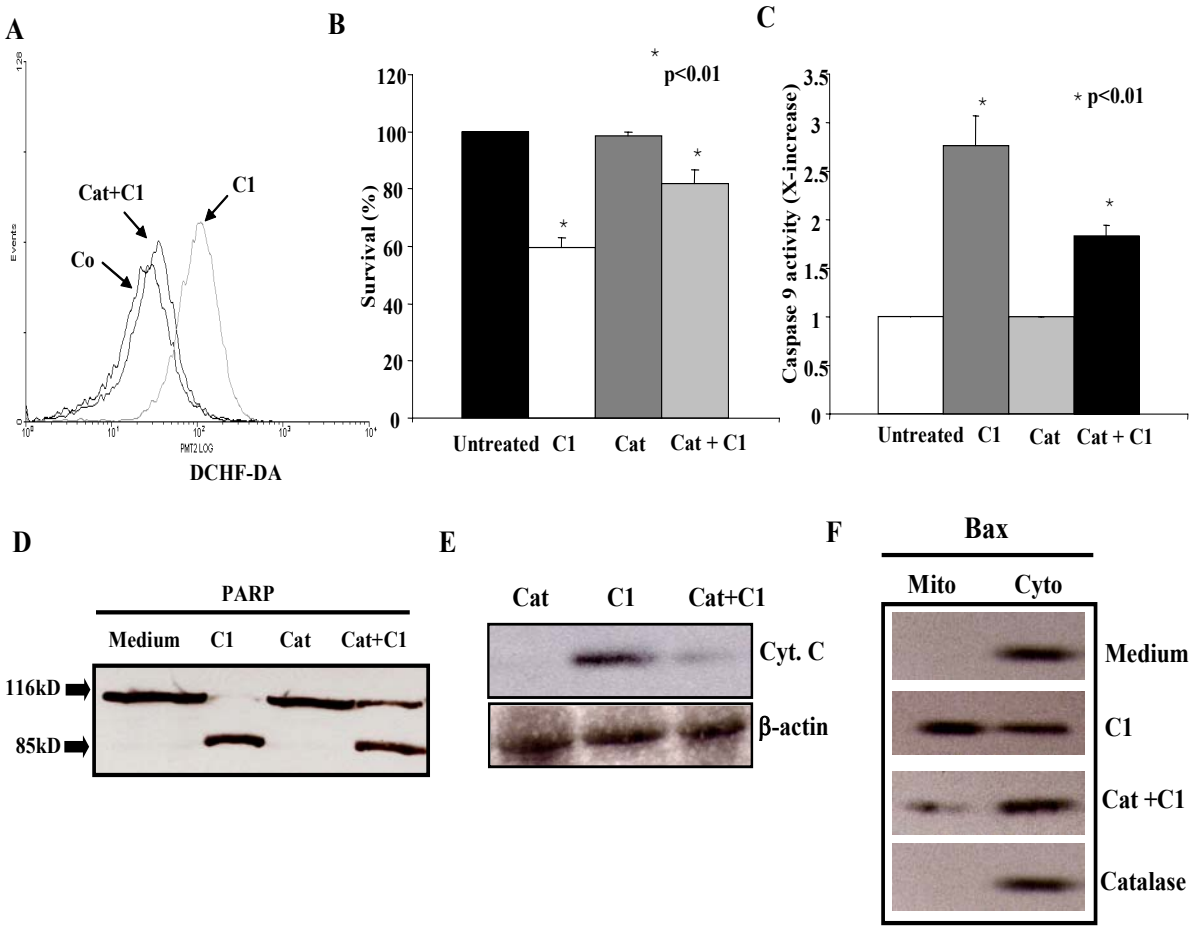

A
Cat+C1
C1
Co
Events
DCHF-DA
B
p<0.01
Survival (%)
Untreated
C1
Cat
Cat + C1
C
p<0.01
Caspase 9 activity (X-increase)
Untreated
C1
Cat
Cat + C1
D
PARP
Medium
C1
Cat
Cat+C1
116kD
85kD
E
Cat
C1
Cat+C1
Cyt. C
β-actin
F
Bax
Mito
Cyto
Medium
C1
Cat +C1
Catalase

Figure-24: Apoptosis and Bax translocation induced by C1 is mediated by intracellular H_2O_2.

HL60 cells ($1x10^6$/ml) were exposed to 50μg/ml of C1 in the presence or absence of 1000U/ml of catalase for 4 hrs (for intracellular H_2O_2), 12 hrs (for caspase activation and Cyt. C translocation),and 18 hrs for cell survival.

(A) Intracellular H_2O_2 was detected by flow cytometry using DCHF-DA as described in Materials and Methods.

(B) Caspase 9 activity was assayed using a fluorogenic substrate (LEHD-fmk) and is shown as fold increase (X increase) over that of the untreated cells. Data are Mean± S.D. of three independent experiments and the significance was calculated by the paired *t*-test.

(C) Lysates obtained from $2x10^6$ cells following exposure to 50μg/ml of C1 for 18 hrs in the presence or absence of catalase were run on 10% SDS-PAGE and western blot analysis for cleavage of PARP was performed using a monoclonal anti-PARP antibody.

(D) Cytosolic fractions were subjected to western blot analysis for Cyt.C translocation.

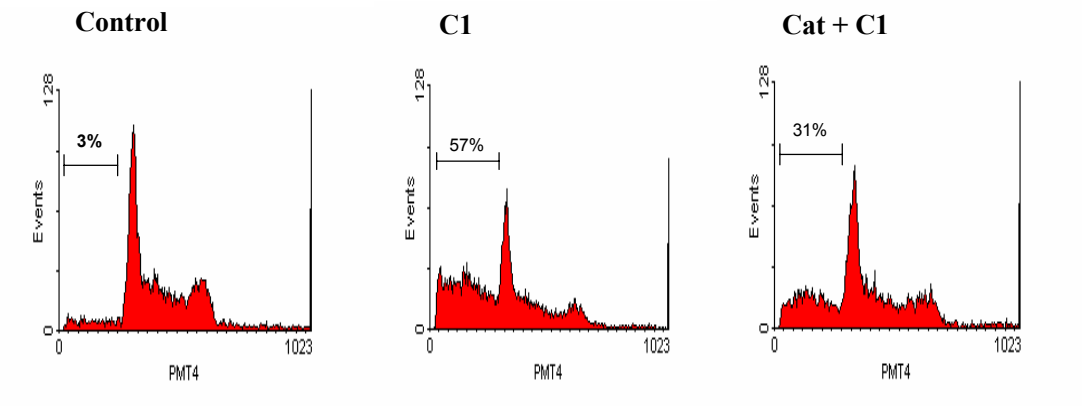


Figure-25: Catalase inhibits apoptosis triggered by C1.

DNA fragmentation was determined by PI staining and the appearance of sub-G1 fraction. $1x10^6$ cells were treated as in B, permeablized and stained with PI as described in Materials and Methods. Cells were analyzed by flow cytometry (10,000 events) and percentage shown are apoptotic cells in sub-G1.

4.2.3 H_2O_2 or C1 induced Bax translocation is independent of CD95 receptor signaling:

We had discussed as part of introduction to this thesis the importance of CD95 signaling (Krammer, 2000)

Having established that Bax translocation upon exposure to C1 was dependent upon intracellular H_2O_2 production, I next focused on the mechanism underlying H_2O_2-mediated Bax translocation during drug-induced apoptosis. As H_2O_2 has been shown to signal via upregulation of the death receptor CD-95, I first questioned the involvement of the CD95 signaling pathway in H_2O_2 and C1-induced apoptosis. The experiment here shows that exposure of HL-60 cells to H_2O_2 or C1 failed to induce upregulation of the CD95 receptor **(Fig-26A-B)**. In addition, pre-incubation of cells with blocking antibody (anti-CD95 IgG) had no effect on cell death triggered by H_2O_2 or C1 **(Fig-26C)**. Furthermore, similar to H_2O_2, incubation of cells with the general caspase inhibitor zvad-fmk did not block Bax translocation triggered by C1 **(Fig-26D)** further consolidating the absence of receptor involvement in this system.

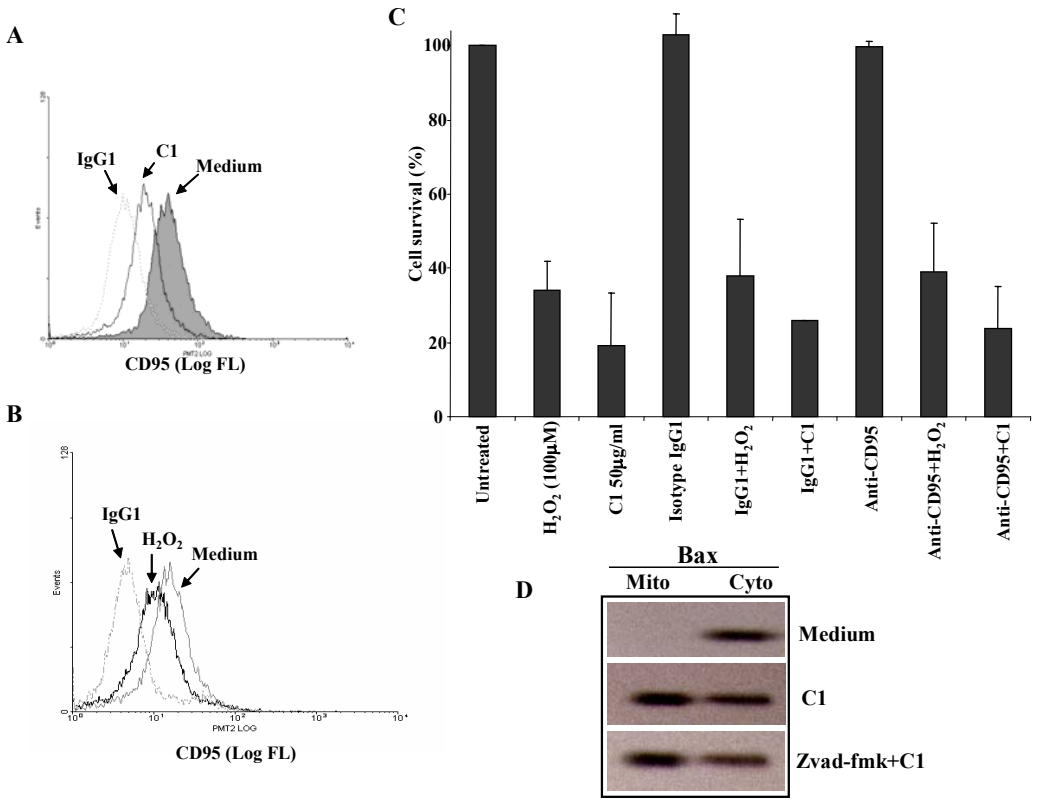


Figure-26: C1 or H_2O_2-induced Bax translocation is independent of CD-95 signaling.

HL60 cells (1X106) were exposed to

(A) 50µg/ml of C1 or **(B)** 100µM H_2O_2 for 12 hours, followed by flow cytometry analysis of CD95 receptor expression as described in Materials and Methods.

(C) HL60 cells (1X106) were pre-incubated with 2ug/ml of anti-CD95 (IgG) for 1 hour prior to triggering apoptosis with C1 or H_2O_2 for 18 hours. Cell survival was assessed by MTT assay. Isotype 1gG is used as a background control.

(D) Mitochondrial and cytosolic fractions from HL60 cells ($2.5x10^7$) following exposure to 50µg/ml of C1 for 12 hours in the presence or absence of ZVAD-fmk (50µM) were subjected to western blot analysis using anti-Bax as described in Materials and Methods.

4.2.4 Drug-induced Bax translocation is H_2O_2 dependent but does not involve the ceramide pathway:

In order to gain further insight into the mechanism underlying Bax translocation, I investigated the involvement of another known inducer of mitochondrial recruitment of Bax, i.e. the membrane sphingomyelinase resulting in intracellular ceramide production (Kim et al., 2001; von Haefen et al., 2002). In order to investigate the role of intracellular ceramide production in H_2O_2 and C1-induced translocation of Bax, HL60 cells were incubated with the sphingomyelinase inhibitor (SMase I; gentamycin sulfate) prior to incubation with C1 (50ug/ml) or 100uM of H_2O_2 for 12 hrs. Pre-incubation with SMaseI had no effect on H_2O_2 or C1-induced Bax translocation thus ruling out that ceramide production is involved in Bax translocation to the motochondria.**(Fig-27**).

4.2.5 Drug-induced Bax translocation does not require new protein synthesis:

In order to decipher the involvement of any new protein synthesis in drug-induced Bax translocation, I next analyzed the effect of protein synthesis inhibitor, cycloheximide (Cyclohex.), on apoptosis and Bax translocation induced by C1 and H_2O_2. The results show that pre-incubation with Cylohex. had no effect on the sensitivity of cells to apoptosis triggered by either stimulus **(Fig-28A)**. More importantly, Bax translocation was not affected by Cyclohex. treatment **(Fig-28B)**, thus ruling out the requirement for new protein synthesis in C1 or H_2O_2 -induced Bax translocation in this system. The findings with cycloheximide and its null effect on

Bax activation have been previously reported but translocation of Bax had not been studied. (Lee et al., 2000)

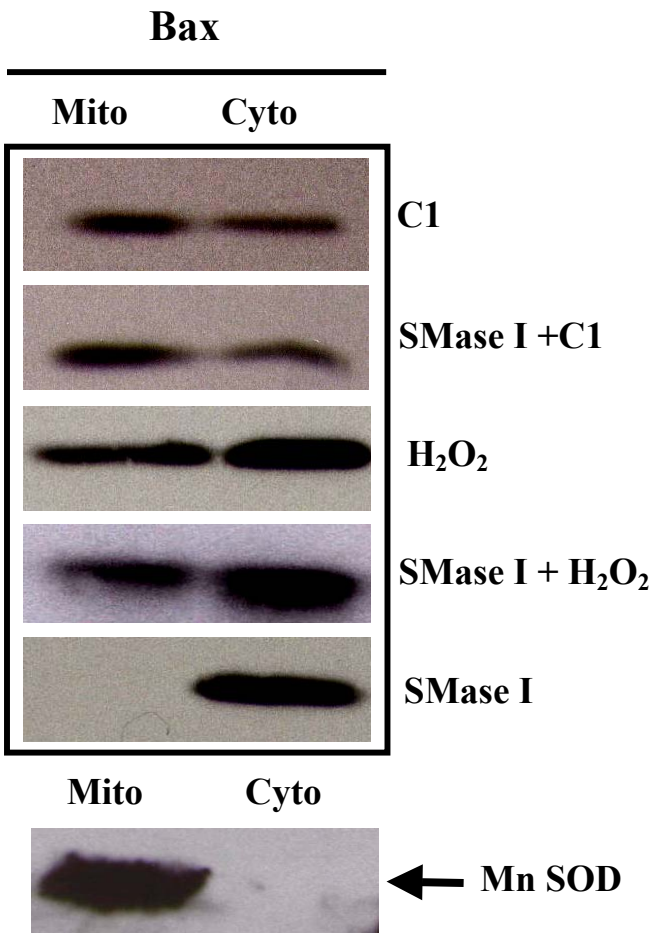


Figure-27: C1 or H_2O_2-induced Bax translocation is independent of intracellular ceramide.

HL-60 cells were treated with C1 or H_2O_2 for 12 hrs in the presence or absence of the sphingomyelinase inhibitor (SMaseI) and mitochondrial and cytosolic fractions were probed by western blot analysis using anti-Bax as described before. The purity of the mitochondrial fraction was confirmed by probing the membrane with anti-MnSOD.

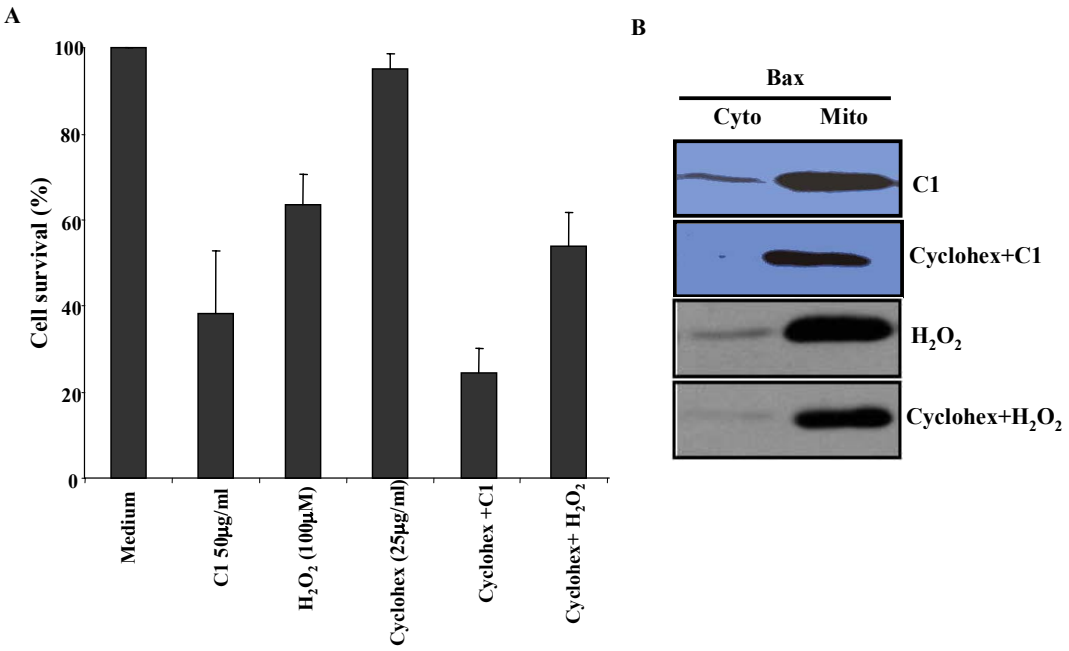


Figure-28: C1 or H_2O_2 induced Bax translocation does not require new protein synthesis.

(A) HL60 cells ($1X10^6$) were pre-incubated with 25ug/ml of Cyclohex. for 4 hours prior to triggering apoptosis with C1 or H_2O_2 or 18 hours. Cell survival was assessed by MTT assay.

(B) Mitochondrial and cytosolic fractions from HL60 cells ($2.5x10^7$) following exposure to 50µg/ml of C1 or 100uM H_2O_2 for 12 hours in the presence or absence of Cyclohex. (25µg/ml) were subjected to western blot analysis using anti-Bax as described in Materials and Methods.

4.2.6 H_2O_2 - signals mitochondrial recruitment of Bax via cytosolic acidification:

Our group has previously demonstrated that H_2O_2 added exogenously or triggered endogenously during drug-induced apoptosis is a strong stimulus for cytosolic acidification, thereby creating a permissive intracellular milieu for death execution (Hirpara et al., 2001). In agreement with earlier reports, these experiments show that exposure of cells to C1 or H_2O_2 resulted in a significant drop in cytosolic pH (pHi), which could be blocked by the H_2O scavenger catalase **(Fig-29A-B)**. I therefore questioned whether H_2O_2-dependent cytosolic acidification induced by C1 could be the stimulus for Bax translocation to the mitochondria. Next to verify that cytosolic pH drop was indeed a signal for Bax recruitment to the mitochondria, I assessed the effect of enforced intracellular acidification on Bax translocation. To do so, I made use of the pharmacological inhibitor of the pH regulator Na+/H+ antiporter (NHE), methylamiloride, and studied its effect on intracellular localization of Bax. As expected, incubation of cells with methylamiloride (10-40uM) resulted in a significant drop in cytosolic pH **(Fig-29C)**. Most interestingly, exposure of tumor cells to amiloride alone resulted in a significant translocation of Bax to the mitochondria **(Fig-29D)**, which was followed by cell death **(Fig-29E)**. This data provide strong evidence to support intracellular pH drop resides downstream of H_2O_2 production and is imperative for the recruitment of Bax during drug-induced apoptosis of tumor cells.

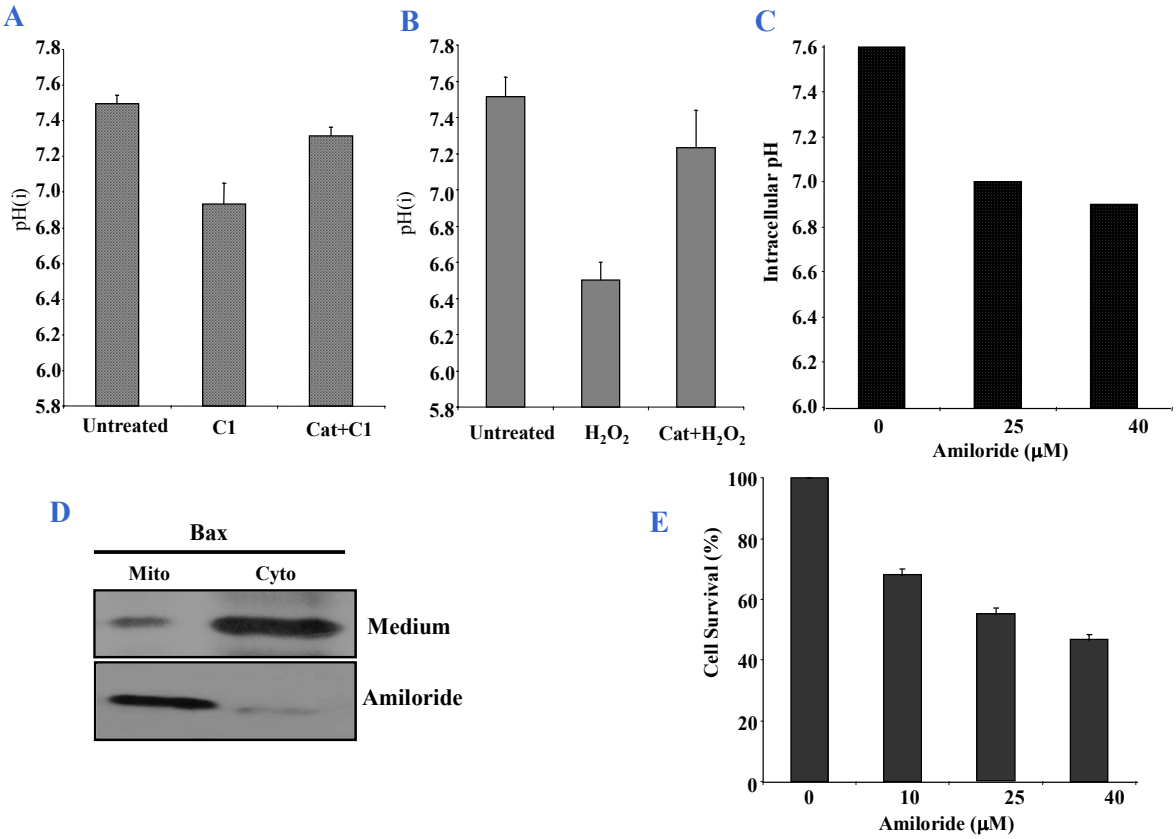


Figure-29: Enforced acidification triggers Bax translocation.

(A) (B) (C) HL60 cells ($1X10^6$) were treated with C1 (50ug/ml) H_2O_2 (100uM) in the presence or absence of 1000U/ml of catalase, and 0-40μM methylamiloride. pH was determined by BCECF-AM loading as described in materials and methods.

(D) HL60 cells ($10X10^6$) were purified for mitochondrial and cytosolic fractions and Bax translocation was detected by Western blotting as described in Materials and Methods.

(E) HL60 cells ($1X10^6$) were treated with 0-40um of Amiloride. Cell survival was assessed by the MTT assay, as described in Materials and Methods.

4.2.7 H_2O_2-induced apoptotic signaling is blocked in Bax-/- cells:

In order to further substantiate my findings to establish a direct relationship between Bax and H_2O_2-induced death signaling, I next assessed the sensitivity of human colon carcinoma cell lines HCT116 Bax+/- and HCT116 Bax-/- **(Fig-30A)** to H_2O_2 (100μM) for 4 to 24 hours. HCT116 Bax-/- cells were completely resistant to the effect of H_2O_2, unlike Bax +/- cells as shown in **(Fig-30B)**. The death inducing activity of H_2O_2 in HCT116 Bax+/- cells was mediated by an early induction (within 4 hours of exposure) of caspases 9 and 3 activities with no significant activation of upstream caspase 8 or cleavage of Bid **(Fig-30C)** , cleavage of the caspase 3 substrate PARP **(Fig-30D).** Similar treatment with H_2O_2 had no significant effect on caspase activation as shown by the absence of PARP cleavage, or DNA fragmentation in HCT116 Bax-/- cells **(Fig-30D, 31A)**. In order to further elucidate the dependence of H_2O_2 death signaling on Bax, we asked if the mitochondrial pathway was critical for the execution of the death signal triggered by exposure to H_2O_2. Indeed, in addition to the significant augmentation in caspase 9 activity in HCT116 Bax+/- cells, H_2O_2 exposure also induced cytosolic translocation of Cyt. C in HCT116 Bax+/- cells (inhibitable by catalase), but not in the Bax-/- variant **(Fig-31C**) Furthermore, transient transfection of Bax -/- cells with a vector containing full-length Bax restored the sensitivity of HCT116 cells to H_2O_2-induced apoptosis **(Fig-31B)**. Confocal images were also obtained by immunostaining for cytochrome C to re-confirm the resistance offrered by Bax -/- cells on H_2O_2 treatment **(Fig-32)**. These data clearly demonstrate that the complete absence of Bax endowed HCT116 cancer cells with the

ability to resist apoptosis triggered by H_2O_2 through mechanism(s) that may hinder the recruitment of the mitochondrial death pathway.

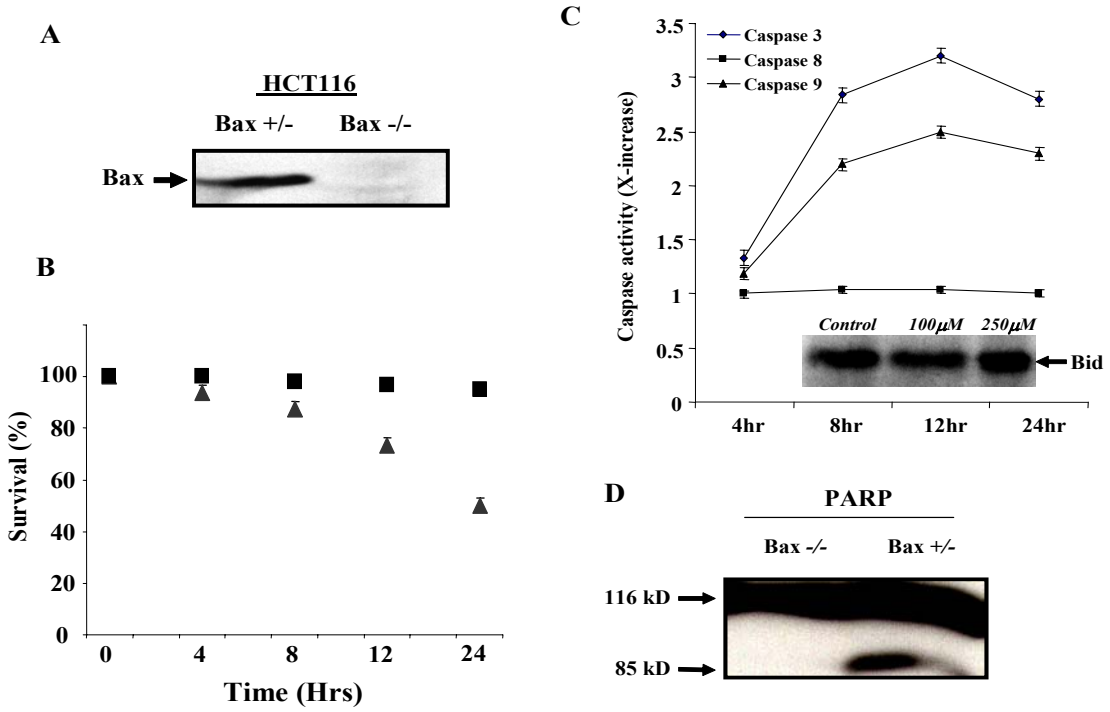


Figure-30: HCT116 Bax knockout (-/-) cells are resistant to H_2O_2-induced apoptosis.

(A) Lysates from HCT116 Bax+/- and Bax-/- cells ($2x10^6$) were subjected to 10% SDS-PAGE and the presence or absence of Bax was confirmed by western blot analysis using monoclonal anti-Bax as described in Materials and Methods.

(B) HCT116Bax+/- and Bax-/- cells ($1x10^6$/ml) were exposed to 100μM H_2O_2 for 4-24 hrs and cell survival was assessed by the MTT assay. Data shown are Mean±S.D. of three independent experiments.

(C) Lysates obtained from H_2O_2-treated cells were analyzed for caspases 3, 8, and 9 activities using fluorogenic substrates as described in Materials and Methods. Results are shown as fold increase (X increase) in caspase activity over the untreated cells (1X). Mean±S.D. of at least three independent experiments is shown.

(D) Western blot analysis of cleavage of the caspase 3-substrate PARP (using anti-PARP clone C-2-10; Pharmingen) in lysates obtained from Bax+/- or Bax-/- cells following 24 hrs incubation with 100μM H_2O_2.

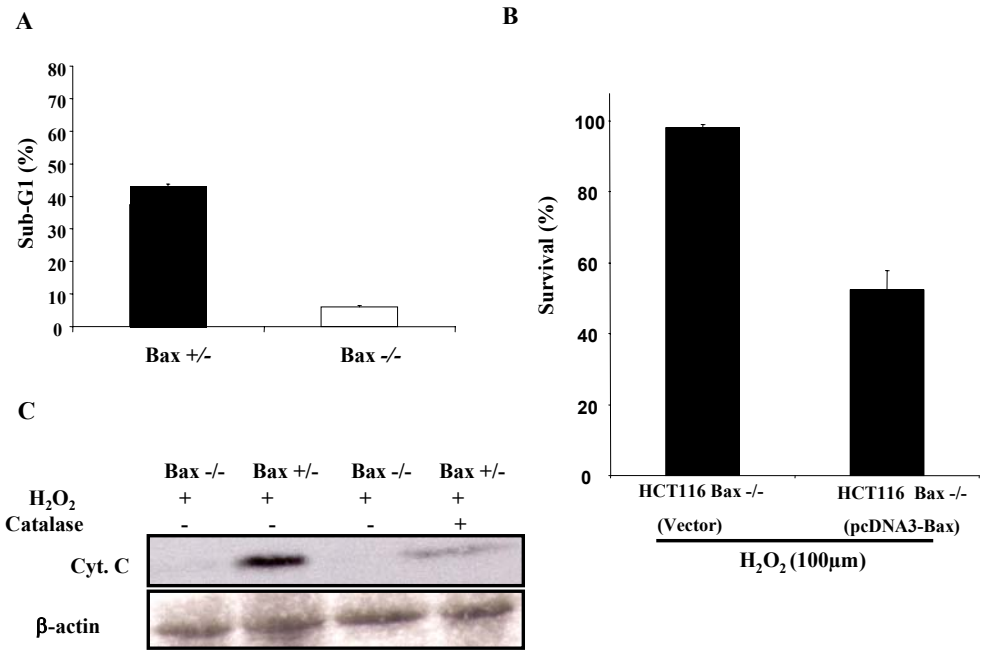


Figure-31: Sensitivity of HCT116 cells to H_2O_2-induced apoptosis is Bax dependent.

(A) Bax+/- and Bax-/- cells were anlayzed for DNA fragmentation by PI staining following 24 hrs treatment with 100µM H_2O_2 as described in Materials and Methods. Sub-diploid population (Sub-G1 fraction), indicative of apoptotic DNA fragmentation, is shown as Mean±S.D. from three independent experiments.

(B) HCT116Bax-/- cells were transiently transfected with pcDNA-3 Bax as described in Materials and Methods and exposed to 100µM H_2O_2 for 24 hrs. Cell survival was assessed by the β-gal survival assay described in Materials and Methods. Data shown are Mean ±S.D. of three independent experiments.

(C) Western blot analysis of Cyt. C in cytosolic fractions of Bax+/- and Bax-/- cells following 12hr treatment with 100µM H_2O_2 in the presence or absence of 1000U/ml of catalase. Membrane was reprobed with anti-β actin as a loading control.

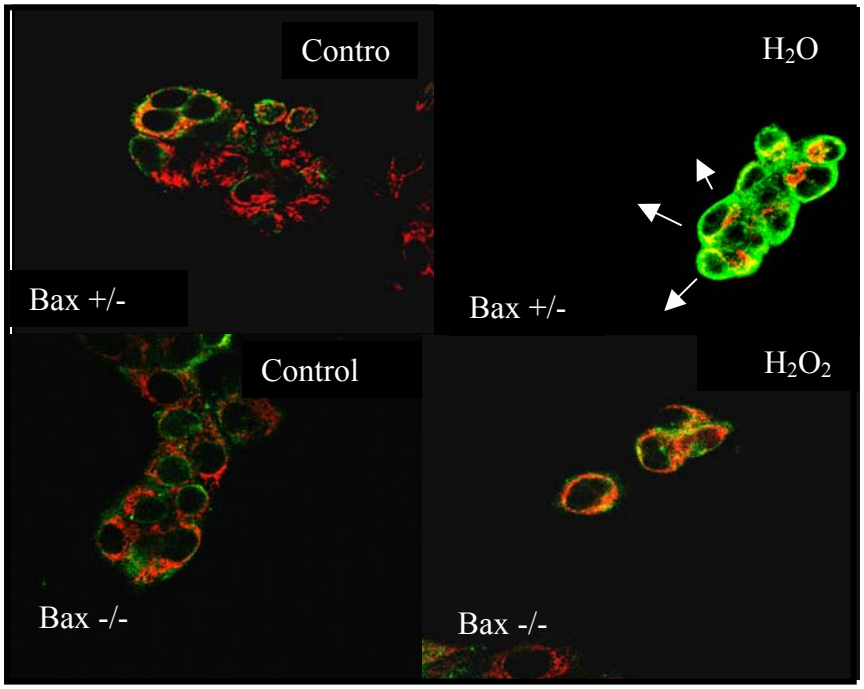


Figure-32: Downstream cytochrome C release is absent in Bax knockout cells in response to H_2O_2.

HCT116 Bax +/- and Bax-/- were plated on coverslips ($0.5x10^5$/ml) a day prior to exposure to H_2O_2 and then imuno-blotted with cytochrome C anti-body 1:100 dilution as primary antibody and then with secondary FITC at 1:50 dilution . The colonic cancer cells were then analyzed by confocal microscope for cytochrome C release as described in Materials and Methods.

4.2.8 H_2O_2 does not influence Bax expression but its intracellular distribution:

Since the background cell line I used was HCT116 and in order to ascertain if the mitochondrial translocation of Bax by H_2O_2 was not exclusive to leukemia cells, I also investigated the effect of H_2O_2 on colonic carcinoma cells to re-confirm my findings on Bax translocation.

Earlier reports have demonstrated that exposure to H_2O_2 results in upregulation of Bax and a shift in the intracellular Bax to Bcl-2 ratio in some systems(Kang et al., 2002; Nakamura and Sakamoto, 2001). Having shown that H_2O_2-induced apoptosis was dependent upon Bax, I next investigated if indeed the sensitivity of HCT116 Bax+/- cells to H_2O_2 was a result of upregulation of Bax expression or a change in the cellular ratio of Bax to Bcl-2. Whole lysates from HCT 116 Bax+/- treated with 100μM H_2O_2 for 0 to 24 hours were subjected to western blot analysis using a monoclonal anti-Bax IgG (1:2500 dil) or anti-Bcl-2 (1:5000dil). Exposure to H_2O_2 did not significantly change the protein levels of Bax or Bcl-2, and consequently their intracellular ratio, in HCT 116 Bax+/- cells as determined by western blot analysis **(Fig-33A**).

In order to investigate the recruitment of Bax, sub-cellular fractions were prepared from HCT 116 Bax+/- cells following exposure to 100μM H_2O_2 for 0-12 hours and the sub-cellular distribution of Bax was assessed by western blot analysis using anti-Bax IgG. Interestingly, exposure of HCT116 Bax+/- cells to H_2O_2 resulted in the recruitment of Bax to the mitochondrial fraction similar to that observed in leukemia

cells. This redistribution of Bax could again be inhibited upon incubation with the H_2O_2 scavenger catalase (1000U/ml), further supporting the role of H_2O_2 in the mitochondrial translocation of Bax. In addition, pre-incubation of cells with the general caspase inhibitor zvad-fmk (50uM) did not inhibit H_2O_2-induced Bax translocation **(Fig-33B)**.

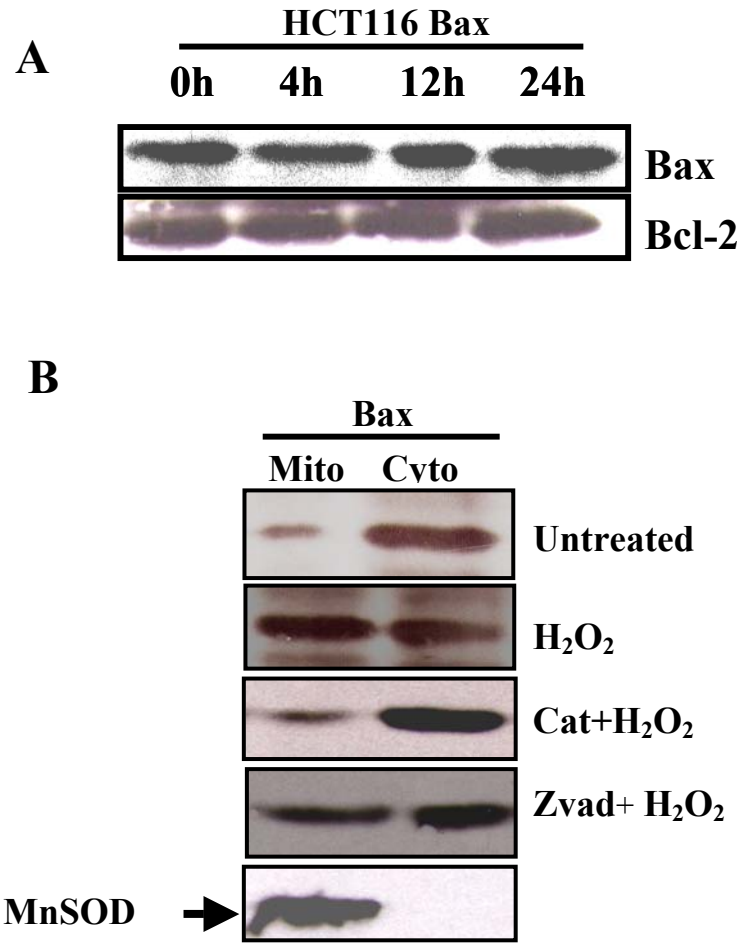


Figure-33: H_2O_2 triggers Bax translocation in HCT116 Bax +/- but does not change Bax/Bcl-2 expression.

(A) Total cell lysates derived from HCT116Bax+/- cells ($5x10^6$) following exposure to H_2O_2 for 0-24 hrs were subjected to western blot analysis using anti-Bax or anti-Bcl-2 antibodies as described in Materials and Methods.

4.2.9 Translocation of Bax triggers further increase in mitochondrial H_2O_2 production:

Current opinion holds that the principal intracellular source of ROS, including H_2O_2, during apoptotic signaling is the mitochondria (Esposti et al., 1999; Fleury et al., 2002; Hirpara et al., 2001). How does one reconcile with these findings given our observations that H_2O_2 acts upstream of the mitochondria to signal translocation of Bax to mitochondrial membranes? In order to address this issue we conducted a simple experiment to ascertain if indeed there was a second wave of intracellular H_2O_2 secondary to Bax translocation. HCT116 Bax+/- and Bax-/- cells were exposed to 100uM H_2O_2 and the intracellular level of H_2O_2 was determined at 30 min, 1hr, 6hrs, and 12hrs by the change in DCF fluorescence by flow cytometry. The initial increase in fluorescence observed in both Bax+/- and Bax-/- cells is indicative of cellular uptake of exogenously added H_2O_2 **(Fig- 34 A panel a**). However, by 1hr, the intracellular level of H_2O_2 (DCF fluorescence) returned to the baseline in Bax-/- cells and remained unchanged for 6 hrs or 12hrs following exposure to H_2O_2 **(Fig- 34 A panels b and c)**. On the contrary Bax+/- cells showed a subsequent increase in intracellular H_2O_2 starting at 6 hrs and significantly detectable even after 12 hrs of the initial exposure to H_2O_2 **(Fig-34 A panels b and c)**. Indeed, a summary of three independent experiments showing change (% of control cells) in mean fluorescence intensity (MFI) of DCF clearly demonstrates a significant increase in MFI at 6 and 12 hrs following exposure to H_2O_2 in Bax+/- cells, whereas no detectable difference is seen in Bax-/- cells **(Fig- 34 A panel a**). It is important to point out that the translocation of Bax to the mitochondria is also detected first at 6 hrs following

incubation with H_2O_2 with a maximum shift observed at 12 hrs post-incubation (Figure 10C) These results suggest a scenario where exogenous or intracellular production of H_2O_2 could trigger Bax translocation, which could act as a signal itself for the second wave of H_2O_2 production from the mitochondria. Similar findings have been observed in neuronal cells where the group has shown that Bax induces a production of H_2O_2 that is critical for apoptotic execultion (Kirkland et al., 2002)

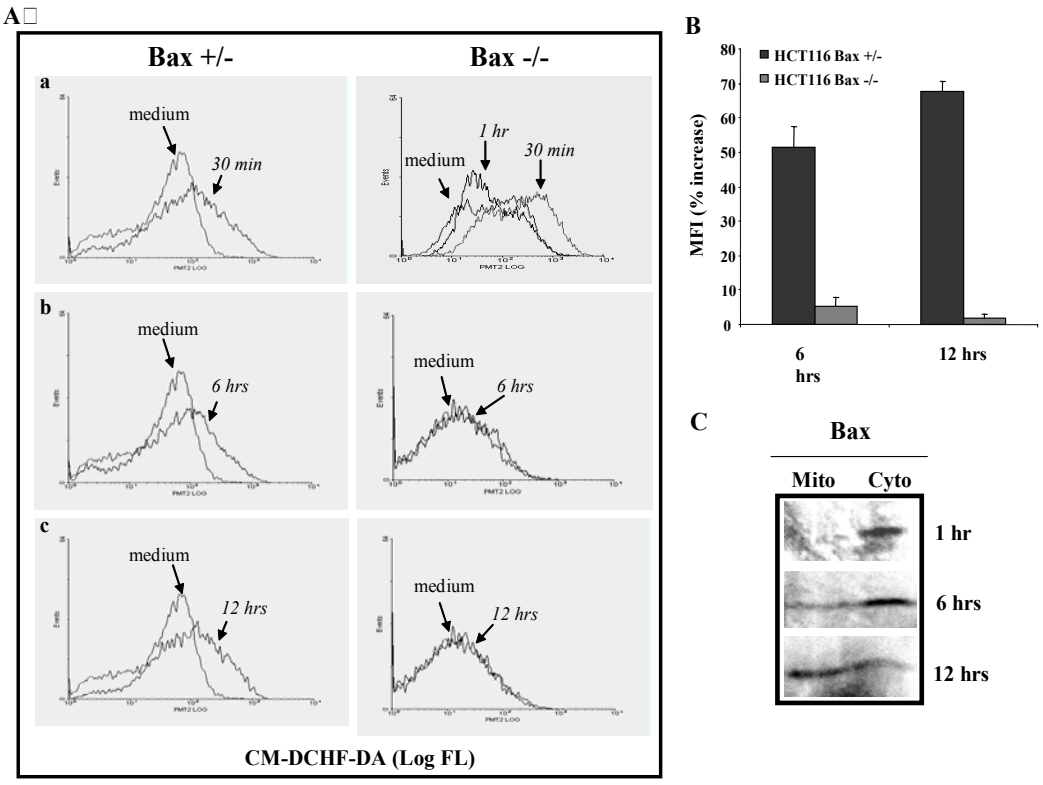


Figure-34: Mitochondrial translocation of Bax results in further increase in intracellular H_2O_2.

(A) HCT116Bax+/- and Bax-/- cells ($1x10^6$) were exposed to H_2O_2 (100μM) and intracellular levels of H_2O_2 were measured at 30min, 1hr, 6hrs, and 12hrs by flow cytometry as described in Materials and Methods.

(B) The Mean Fluorescence Intensity (MFI) indicative of the mean intracellular H_2O_2 levels from three independent experiments, performed on Bax+/- and Bax-/- cells, is plotted to demonstrate the increase in intracellular H_2O_2 only in Bax+/- cells.

(C) Bax+/- cells ($1x10^7$) were incubated with 100μM H_2O_2 for 1hr, 6hrs, and 12hrs followed by cell fractionation and western blot analysis using anti-Bax as described in Materials and Methods.

4.2.10 Preface to next series of experiments:

The experiments have so far shown that H_2O_2 production is critical for C1- induced apoptosis. Furthermore, the experiments demonstrate importance of H_2O_2 signaling through a drop in pH that recruits Bax to the mitochondria eventually leading to mitochondrial apoptosis. Bax translocation may lead to generation of more H_2O_2 forming a positive feedback loop to amplify or re-inforce the death signal. I next asked the importance of {H_2O_2 >> pH >> Bax} circuitry in chemotherapy induced cell death of tumor cells. Here I made use of initial observations that low doses of compounds such as resveratrol (RSV) could induce resistance in tumor cells. Thus, I investigated if RSV induced inhibition is a function of generating an intrcellular milieu that is non-permissive for efficient apoptotic execution, in particular H_2O_2 mediated signaling.

4.3 Resveratrol and its role in manipulating the redox status of tumor cells: from death to survival.

4.3.1 Low doses of RSV inhibit H_2O_2-induced apoptosis in HL-60 cells:

Before studying the effects of RSV it can be demonstrated that low doses of RSV (4-8uM) is non-toxic to leukemia cells **(Fig-35**).

Corroborating the above findings on the death inducing ability of H_2O_2 in cancer cells, I again performed fresh experiments by exposing HL-60 cells to exogenous

H_2O_2 (100μM) and as expected this resulted in a significant decrease in cell survival **(Fig-36A**). That this cytotoxic activity of H_2O_2 was, indeed, a function of activation of the apoptotic death pathway was again evidenced by the significant increase in caspase 3 activity and cleavage of the caspase 3 substrate PARP **(Fig-36B-C)**. Pre-incubation of cells with low doses of RSV (4-8μM) for 2 hours prior to the addition of H_2O_2 for 18 hours resulted in a significant increase in cell survival **(Fig-36A**), inhibition of caspase 3 activity and the cleavage of the caspase 3 substrate PARP **(Fig-36B-C)**. Furthermore, cell cycle analysis of DNA content demonstrated that pre-incubation with RSV significantly reduced the sub-G1 population upon H_2O_2 treatment **(Fig-36D**). Interestingly, RSV treatment resulted in a significant accumulation of cells in the S phase of the cell cycle, an observation previously reported in human cancer cells upon exposure to RSV (Bernhard et al., 2000; Joe et al., 2002; Ragione et al., 1998). I was intrigued by these findings, particularly in the light of our earlier data and that of others demonstrating the apoptosis inducing activity of RSV in cancer cells (Ahmad et al., 2001; Clement et al., 1998a; Ding and Adrian, 2002; Huang et al., 1999; She et al., 2001). However, in those studies the concentrations of RSV used were at least 10-20 folds higher than those used in the present study.

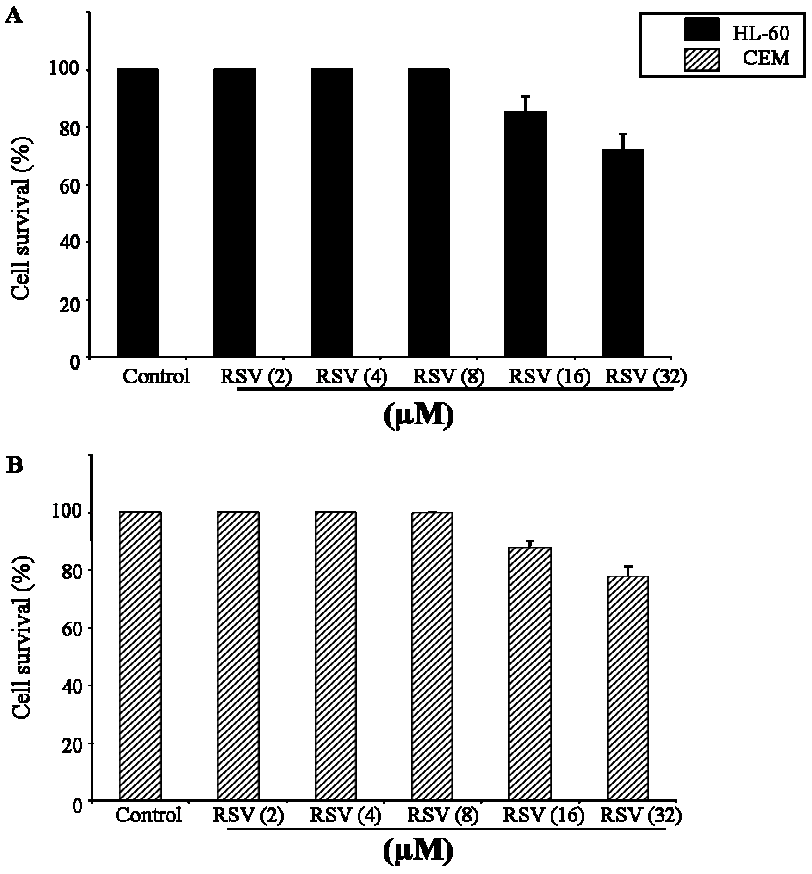


Figure 35: RSV at low doses is non-toxic to tumor cells.

(A) HL-60 cells and **(B)** CEM cells ($1x10^6$cells/mL) were incubated were treated with different doses of RSV for 18h. Cell viability was determined by MTT assay as described in Materials and Methods

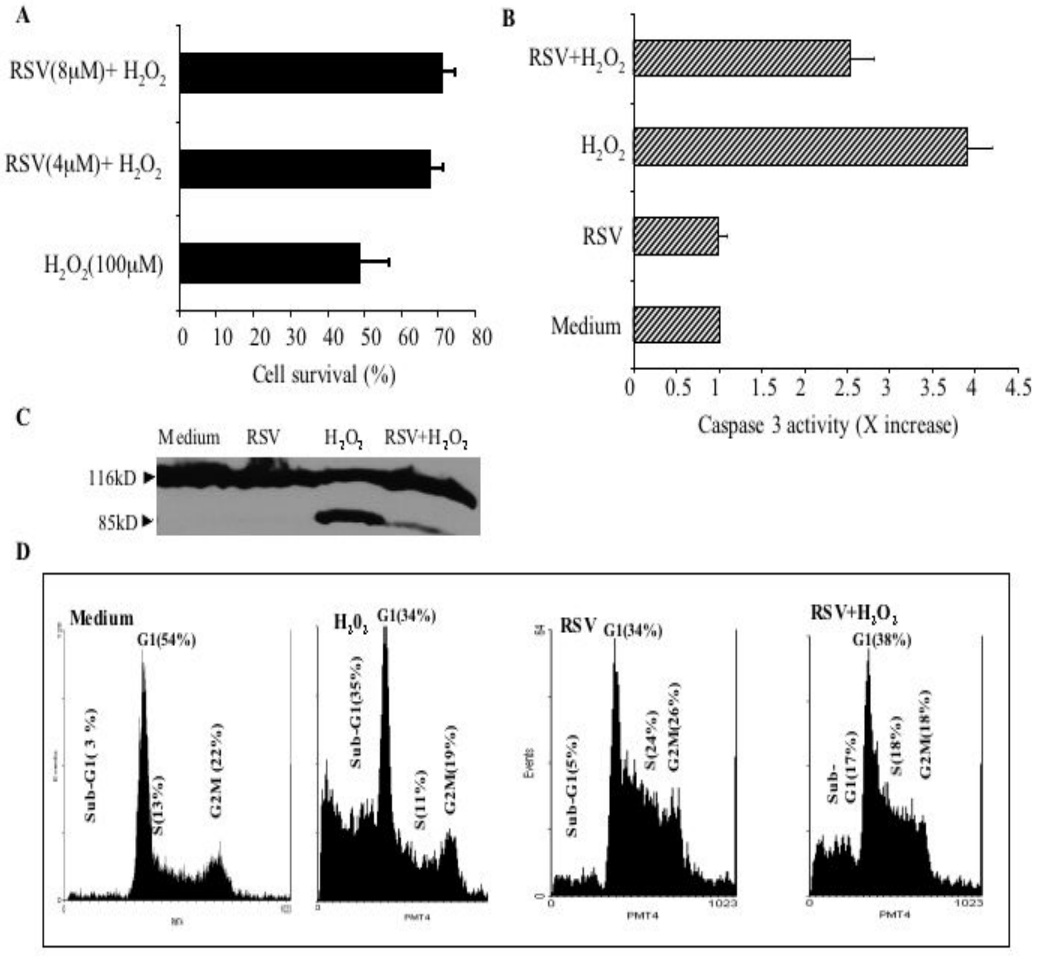

A
RSV(8μM)+ H2O2
RSV(4μM)+ H2O2
H2O2(100μM)
0 10 20 30 40 50 60 70 80
Cell survival (%)
B
RSV+H2O2
H2O2
RSV
Medium
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
Caspase 3 activity (X increase)
C
Medium RSV H2O2 RSV+H2O2
116kD
85kD
D
Medium
G1(54%)
Sub-G1(3 %)
S(13%)
G2M (22%)
H2O2 G1(34%)
Sub-G1(35%)
S(11%)
G2M(19%)
RSV G1(34%)
Sub-G1(5%)
S(24%)
G2M(26%)
RSV+H2O2
G1(38%)
Sub-G1(17%)
S(18%)
G2M(18%)
PMT4
1023

Figure-36: Pre-incubation with RSV inhibits H_2O_2-induced caspase 3 activation and DNA fragmentation in human leukemia cells.

(A) HL60 ($1x10^6$ cell/ml) were incubated with 100μM H_2O_2 for 18 hours or with RSV (4 and 8 μM) for 2 hours followed by similar exposure to H_2O_2. Cell viability was determined by the MTT assay as described in Materials and Methods. Data are shown as Mean S.D. of at least three independent experiments performed in triplicates.

(B) Caspase 3 activity was determined in cell lysates obtained from $2x10^6$ cells treated for 12 hours with 100μM H_2O_2 with or without pre-incubation for 2 hours with RSV (8μM) by a fluorimetric assay using the caspase 3 specific substrate DEVD-AFC. Enzymatic activity was detected by a spectrofluorimeter as described in Materials and Methods and is shown as fold increase (X increase) in activity over that obtained from lysates of untreated cells (1X). Data are the Mean S.D. of three independent experiments.

(C) Cleavage of the caspase 3 substrate PARP was assessed by western blot analysis using a polyclonal anti-PARP (clone C2-10) antibody in lystaes of $2X10^6$ cells following exposure to H_2O_2 (18 hours) with or without prior incubation for 2 hours with RSV (8μM). The 85kD band indicates apoptotic cleavage of PARP.

(D) DNA fragmentation was determined by PI staining and the appearance of sub-G1 fraction. $1x10^6$ cells were treated as in (A), permeablized and stained with PI as described in Materials and Methods. Cells were analyzed by flow cytometry (10,000 events) and percentage of cells in the various phases of the cell cycle are shown.

4.3.2 RSV mediated inhibition of H_2O_2 induced apoptosis is upstream of the mitochondria:

Consistent with the data shown previously in this thesis, exposure of HL60 cells to H_2O_2 resulted in a significant drop in the mitochondrial $\Delta\psi m$ **(Fig-37A)**, an early event during apoptosis indicating that the mitochondrial integrity had been compromised. Pre-incubation of cells with RSV prevented the drop in $\Delta\psi m$ triggered by H_2O_2 as shown in **(Fig-37A)**. To confirm that block was upstream of the mitochondria, RSV significantly inhibited H_2O_2-induced cytosolic translocation of Cyt.C from the mitochondria and the subsequent activation of caspase 9 **(Fig-37B-C)**. These data indicate that the inhibitory activity of RSV on H_2O_2 signaling involves mechanism(s) upstream of the mitochondria or signals that engage the mitochondrial death machinery. One possible explanation for the observed inhibitory effect of RSV could be that RSV functioned as an efficient scavenger of H_2O_2. If so, simultaneous exposure of cells to RSV and H_2O_2 should have the same effect as prior incubation with RSV and the subsequent addition of H_2O_2. Interestingly, our data clearly showed that addition of RSV and H_2O_2 simultaneously to the culture medium (*RSV/H_2O_2*) had no effect on H_2O_2 mediated cell death **(Fig-37D)**, however, prior incubation with RSV for 2 hours before the addition of H_2O_2 (RSV+H_2O_2) was required to induce the inhibitory effect of RSV on death signaling **(Fig- 37D)**. These data clearly indicate that the death inhibitory effect of RSV is not simply a function of its anti-oxidant activity or the ability to scavenge H_2O_2, but involves mechanism(s) that negatively affect critical pathways involved in apoptotic execution.

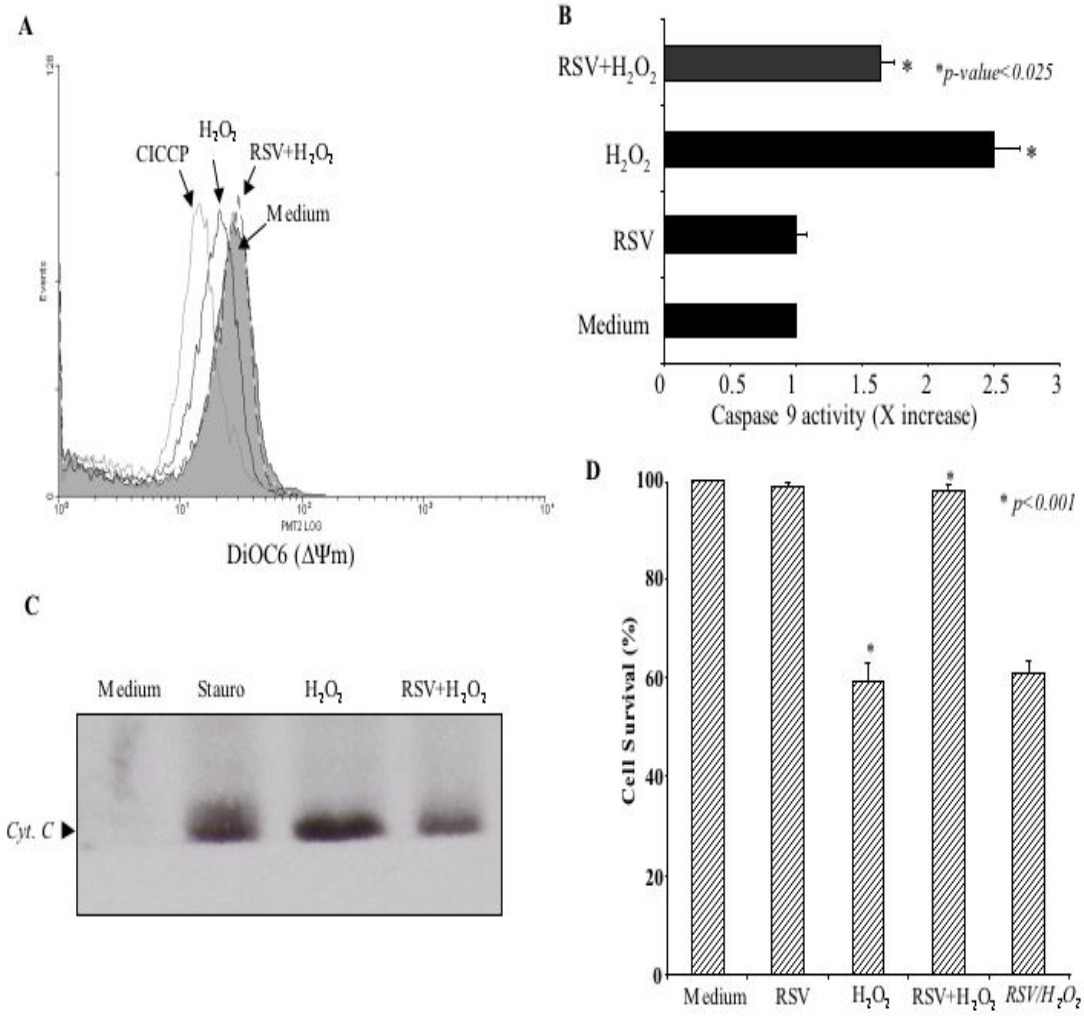

A
CICCP
H_2O_2
RSV+H_2O_2
Medium
Events
PMT2 LOG
DiOC6 (ΔΨm)
B
RSV+H_2O_2
H_2O_2
RSV
Medium
*p-value<0.025
0
0.5
1
1.5
2
2.5
3
Caspase 9 activity (X increase)
C
Medium
Stauro
H_2O_2
RSV+H_2O_2
Cyt. C
D
Cell Survival (%)
* p<0.001
Medium
RSV
H_2O_2
RSV+H_2O_2
RSV/H_2O_2

Figure-37: Inhibitory effect of RSV on H_2O_2-induced death signaling is upstream of the mitochondria.

(A) HL60 **(**$1x10^6$) cells were exposed to 100μM H_2O_2 for 4 hours with or without prior incubation for 2 hours with RSV (8μM) followed by loading of cells with the mitochondrial membrane potential-sensitive dye DiOC6 (40nM) as described in Materials and Methods. As a positive control, cells were exposed to the uncoupler CICCP (100μM) for 30 minutes. $\Delta\psi_m$ was determined by flow cytometry with the excitation set at 488 nm. At least 10,000 events were analyzed for each set of experiment.

(B) Cell lysates from $2x10^6$ cells treated with 100μM H_2O_2 (12 hours) with or without prior incubation for 2 hours with RSV (8μM) were subjected to a fluorimetric assay designed to detect caspase 9 activity (caspase 9 specific substrate LEHD-AFC). Enzymatic activity is shown as fold increase over that obtained from lyates of untreated cells (1X). Data are Mean S.D. of three independent experiments.

(C) Cytosolic translocation of Cyt. C was assessed by western blot analysis of cytosolic extracts from $30x10^6$ HL60 cells treated with H_2O_2 for 12 hours with or without prior incubation with RSV (8μM) as described in Materials and Methods. Staurosporine treated HL60 cytosolic extract was used as a positive control.

(D) Cytosolic pH was determined by loading $1x10^6$ cells with the pH sensitive probe BCECF-AM as described in Materials and Methods. pH values were derived from a standard pH curve obtained from cells cultured in different pH buffers in the presence of nigericin (1μg/ml).

4.3.3 RSV inhibits H_2O_2-induced apoptosis by creating a non-permissive milieu for caspase activation:

Our group has previously demonstrated that apoptosis induced by H_2O_2 is mediated by a decrease in intracellular O_2^- concentration and cytosolic acidification and discussed it briefly in our introduction (Clement et al., 1998b). We described this death as "reductive stress induced apoptosis" in order to distinguish it from the oxidative stress-mediated death affected by necrotic concentrations of H_2O_2 (Pervaiz and Clement, 2002a). Our recent reports and as introduction to this thesis provide evidence that a decrease in O_2^- and cytosolic acidification creates a permissive environment for the execution of the apoptotic signal (Clement et al., 2003; Pervaiz et al., 2001; Pervaiz et al., 1999a). The fact that low concentrations of RSV negatively impacted effector components of the death-signaling circuitry stimulated us to further investigate if the inhibitory effect of RSV was due to its ability to prevent a drop in intracellular O_2^- and cytosolic acidification induced by H_2O_2. In other words, I simply asked if the inhibitory activity of RSV on H_2O_2-induced apoptosis was a function of creating an intracellular milieu non-conducive for efficient death execution. Consistent with our earlier reports, I show here that exposure of HL60 cells to H_2O_2 resulted in a significant decrease in the intracellular concentration of O_2^- **(Fig-38B)**. Not only did RSV induce an increase in intracellular O_2^- concentration, but also completely blocked the inhibitory effect of H_2O_2 on intracellular O_2^- as shown in (**Fig-38B**). Interestingly, pre-incubation with RSV followed by the addition of H_2O_2 consistently resulted in higher intracellular concentration of O_2^- than RSV alone. In addition, cytosolic acidification triggered by H_2O_2 was completely blocked upon prior

exposure to RSV **(Fig-38A)**. These data indicate that the inhibitory effect of RSV on death signaling induced in leukemia cells by H_2O_2 is not due to its anti-oxidant activity, but contrarily is mediated by an increase in intracellular O_2^- level and inhibition of the drop in cytosolic pH. Thus, low doses of RSV result in the generation of a slight pro-oxidant state that has been linked to inhibition of apoptotic signaling in cancer cells, and suggested to provide cancer cells with a survival advantage over their normal counterparts by positively influencing cell proliferation (Burdon, 1995; Cerutti, 1985).

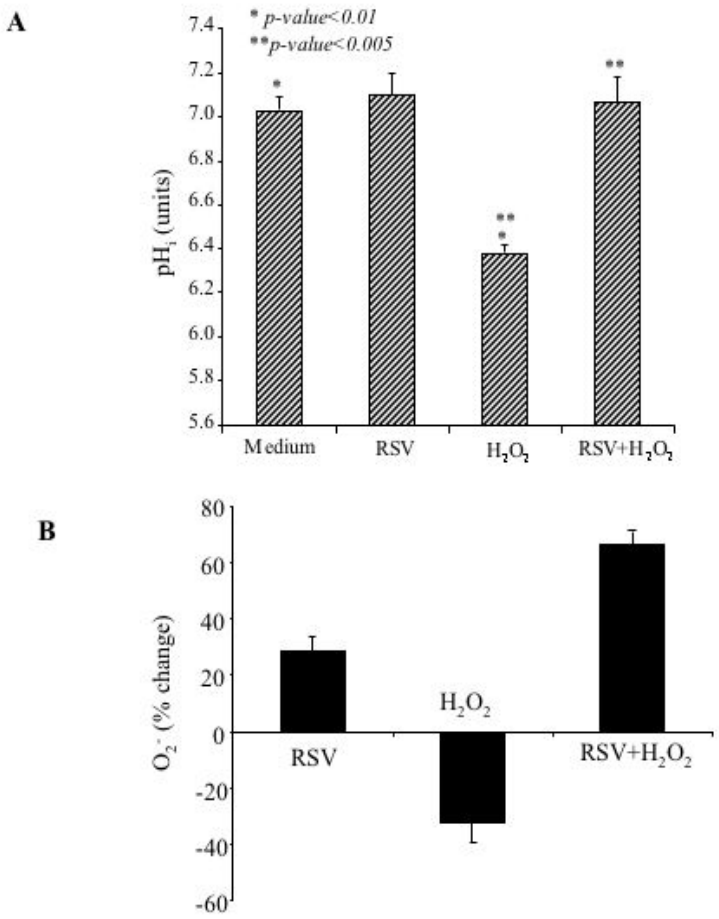


Figure-38: The death inhibitory activity of RSV is not due to its anti-oxidant activity.

(A) HL60 cells ($1x10^6$/ml) were incubated with RSV (8μM) or pre-incubated for 2 hours with RSV (8μM) prior to the addition of 100μM H_2O_2 (RSV+H_2O_2). Cytosolic pH was determined by loading $1x10^6$ cells with the pH sensitive probe BCECF-AM as described in Materials and Methods. pH values were derived from a standard pH curve obtained from cells cultured in different pH buffers in the presence of nigericin (1μg/ml).

(B) $2X10^6$ cells were treated with RSV (8μM) for 2 hours or H_2O_2 (100μM) for 4 hours with or without 2 hours of prior incubation with RSV (8μM) and intracellular O_2^- was measured by a lucigenin-based chemiluminescence assay as described in Materials and Methods. Data are shown as the percentage difference in the intracellular O_2^- concentration compared to untreated control cells and are the Mean S.D of at least three independent experiments.

4.3.4 RSV-induced inhibition of death signaling can be reverted by blocking the activation of NADPH oxidase complex:

The next step was to investigate the mechanism of the increase in intracellular O_2^- affected by RSV. The two main sources of intracellular O_2^- are the membrane-bound NADPH oxidase complex and the mitochondrial electron transport chain (Halliwell and Gutteridge, 1999). Using DPI, a specific inhibitor of the NADPH oxidase complex, we show here that RSV-induced increase in intracellular O_2^- could be completely blocked in the presence of DPI **(Fig- 39A).** Furthermore, incubation of cells for 1 hour with DPI before the addition of RSV did not only restore death signaling triggered by H_2O_2, but indeed significantly enhanced the sensitivity of HL60 and CEM leukemia cells to H_2O_2 **(Fig-39B)**. These results provide evidence that the increase in intracellular O_2^- upon exposure of HL60 cells to RSV was mediated through the activity of the NADPH oxidase complex, and that this increase in intracellular O_2^- was, indeed, linked to the death inhibitory activity of RSV. In order to provide further credence to our findings that this was indeed a function of NADPH oxidase activation, we used a dominant negative mutant of the small GTP binding protein Rac (RacN17) that has been shown to inhibit NADPH oxidase dependent increase in intracellular O_2^-(Pervaiz et al., 2001). Rac is a downstream effector of ^{p21}Ras oncogenic signal, and an increase in intracellular O_2^- has been implicated in this pathway(Diekmann et al., 1994; Irani et al., 1997; Kreck et al., 1994). In order to evaluate the effect of RacN17 on the inhibitory activity of RSV, CEM leukemia cells were transiently co-transfected with either the empty vector (pIRES) or with pIRES RacN17 and a β-gal containing vector. Forty-eight hours

post-transfection, cells were incubated with H_2O_2 as an apoptotic stimulus and cell viability was calculated using the β-gal survival assay described in Materials and Methods. Similar to the results obtained with DPI, transient transfection of leukemia cells with RacN17 completely reverted the sensitivity of cells to exogenous H_2O_2 **(Fig- 39C)**.

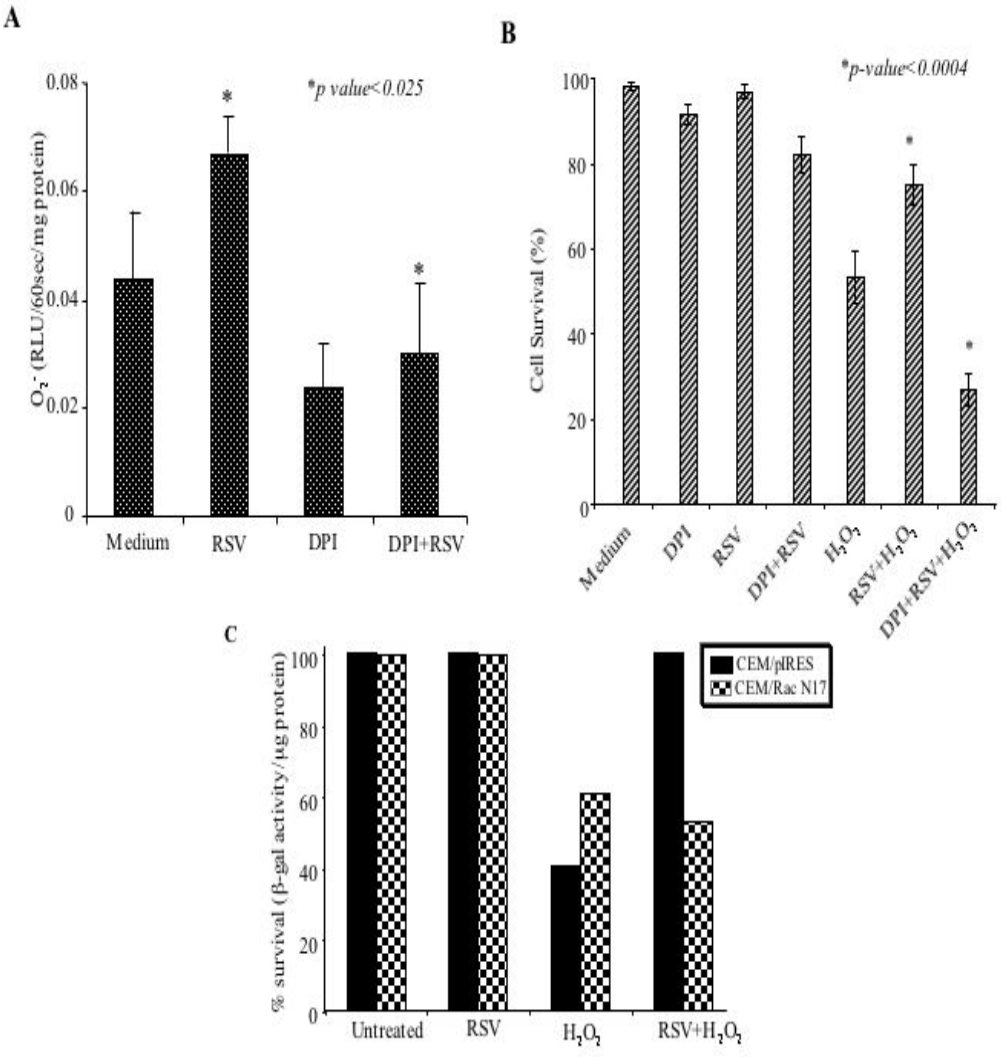

A
*p value<0.025
O_2^- (RLU/60sec/mg protein)
0.08
0.06
0.04
0.02
0
Medium
RSV
DPI
DPI+RSV
B
*p-value<0.0004
Cell Survival (%)
100
80
60
40
20
0
Medium
DPI
RSV
DPI+RSV
H_2O_2
RSV+H_2O_2
DPI+RSV+H_2O_2
C
% survival (β-gal activity/μg protein)
100
80
60
40
20
0
CEM/pIRES
CEM/Rac N17
Untreated
RSV
H_2O_2
RSV+H_2O_2

Figure-39: Inhibition of NADPH oxidase activation prevents RSV-induced increase in intracellular O_2^- and overcomes the death inhibitory effect of RSV.

(A) HL60 ($2X10^6$) cells were incubated with 8μM RSV for 4 hours in the presence or absence of DPI (1.25μM) and intracellular O_2^- was measured as described in Materials and Methods. Data shown are the Mean S.D. of three independent experiments and presented as RLU/60 sec/μg protein).

(B) HL60 cells ($1x10^6$/ml) were pre-incubated with DPI (1.25μM) or with DPI (1.25μM)+RSV (8μM) for 4 hours prior to the addition of 100μM H_2O_2 for 18 hours. Cell survival was assessed by the MTT assay as described in Materials and Methods. Mean S.D. of three independent experiments is shown.

(C) CEM leukemia cells ($1x10^6$/ml) were co-transfected with a β-gal containing vector and either the empty pIRES vector or the pIRES RacN17 vector. Forty-eight hours post transfection, cells were exposed to 100μM H_2O_2 for 18 hours with or without prior incubation for 2 hours with 8μM RSV. Cell survival was assessed by the β-gal survival assay as described in Materials and Method and are presented in percentages as β-gal activity/μg protein. Mean of two transfections done independently is shown.

4.3.5 RSV inhibits apoptosis triggered by novel anti-cancer drug C2:

Having shown that RSV inhibited apoptosis induced by the exogenous addition of H_2O_2, I next investigated the effect of pre-incubation of human leukemia cells to RSV on apoptosis induced by a novel anti-cancer compound that kills by intra-cellular generation of H_2O_2. In our earlier reports we demonstrated that exposure of human leukemia and melanoma cells to C2, a purified photoproduct of MC540, triggered mitochondrial generation of H_2O_2 that was responsible for the release of Cyt. C and downstream activation of the caspase cascade (Hirpara et al., 2001). Consistent with those data, here I show that exposure of HL60 cells to 50μg/ml of C2 for 4 hours resulted in a significant increase in the intracellular levels of H_2O_2, which could be completely scavenged by the addition of catalase **(Fig- 40A)**. Similar to the data shown with H_2O_2, pre-incubation of cells with RSV prior to the addition of C2 resulted in an increase in cell survival compared to the cells treated with C2 alone **(Fig-40B)**). Whereas, C2 alone induced robust activation of caspases 3 and 9, pre-incubation with RSV significantly inhibited enzyme activities and cleavage of the caspase 3 substrate PARP in lysates from HL60 cells **(Fig-40C).** To substantiate that this inhibition was mediated via blocking mitochondria-dependent death signaling was evidenced by the significant decrease in the translocation of mitochondrial Cyt.C to the cytosols of cells pre-incubated with RSV prior to the addition of C2 **(Fig-40D)**. Furthermore, cell cycle analysis of cells exposed to RSV and then C2 showed a significant decline in the proportion of sub-G1 DNA content and accumulation in the S phase of the cell cycle **(Fig-40E)**. This was similar to the results obtained with H_2O_2-induced apoptosis at exposure to low concentrations of RSV prevented

leukemia cells from undergoing drug-induced apoptosis. This effect appears to be mediated via inhibition of caspase activation/activity and/or triggering pathways that favor cellular proliferation. The latter is evidenced by the accumulation of cells in the S phase of the cell cycle, an observation reported earlier in response to RSV (Estrov et al., 2003; Joe et al., 2002; Kuwajerwala et al., 2002; Park et al., 2001a; Ragione et al., 1998). More importantly the death inhibitory effect of RSV was not due to its ability to scavenge intracellular H_2O_2 triggered by exposure to C2. A recent report suggested that drugs can produce H_2O_2 within the growing or culture medium (Halliwell et al., 2000). I had confirmed this by incubating the drug in plain medium used for cell culture loaded it with DCF-DA. Readings were obtained from the spectro-fluorometer for 60 minutes. H_2O_2 (100uM) was used as a positive control The data demonstrates nil production of H_2O_2 in the medium by addition of C2 proving that C2 triggered H_2O_2 production was intra-cellular and not an artifact produced in the medium **(Fig-41)** Thus to conclude the data suggests that the inhibitory effect of RSV on death signaling involved pathways that converge on the mitochondria and facilitate death execution. By implication, this would mean that RSV elicited its inhibitory effect by interfering with intracellular effector mechanism(s) triggered by anti-cancer drug exposure that involve the mitochondrial death machinery.

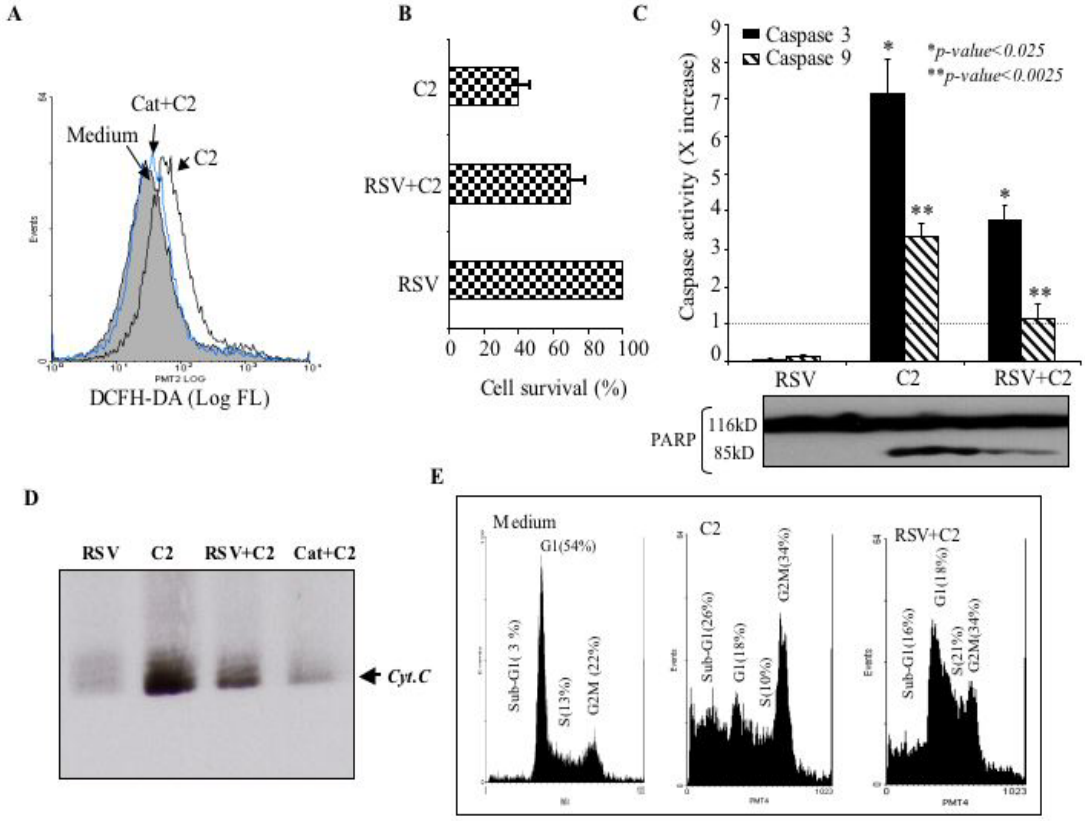

A
Cat+C2
Medium
C2
Events
PMT2 LOG
DCFH-DA (Log FL)
B
C2
RSV+C2
RSV
0 20 40 60 80 100
Cell survival (%)
C
Caspase 3
Caspase 9
*p-value<0.025
**p-value<0.0025
Caspase activity (X increase)
RSV
C2
RSV+C2
PARP
116kD
85kD
D
RSV
C2
RSV+C2
Cat+C2
Cyt.C
E
Medium
G1(54%)
Sub-G1(3 %)
S(13%)
G2M (22%)
C2
Sub-G1(26%)
G1(18%)
S(10%)
G2M(34%)
RSV+C2
Sub-G1(16%)
G1(18%)
S(21%)
G2M(34%)
PMT4

Figure-40: RSV inhibits apoptosis triggered by a novel anti-cancer drug C2 that signals via intracellular production of H_2O_2.

(A) HL60 ($1x10^6$) cells were treated with 50μg/ml of C2 for 4 hours in the presence or absence of 500U/ml of catalase**.** Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5μM) for 30 min. and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry, as described in Materials and Methods.

(B) $1x10^6$ cell/ml were incubated with 50μg/ml of C2 for 18 hours or with RSV (8μM) for 2 hours followed by similar exposure to C2. Cell viability was determined by the MTT assay as described in Materials and Methods. Data are shown as Mean S.D. of at least three independent experiments performed in triplicates.

(C) Activities of caspases 3 and 9 were determined in cell lysates obtained from $2x10^6$ cells treated for 12 hours with 50μg/ml of C2 with or without pre-incubation for 2 hours with RSV (8μM) by a fluorimetric assay using the specific substrates. Enzymatic activities were detected by a spectro-fluoroeter as described above. Cleavage of the caspase 3 substrate PARP was assessed by western blot analysis using a polyclonal anti-PARP (clone C2-10) antibody in lystaes of $2X10^6$ cells following exposure to C2 (18 hours) with or without prior incubation for 2 hours with RSV (8μM). The 85kD band indicates apoptotic cleavage of PARP.

(D) Release of Cyt. C was assessed in cytosolic extracts from $30x10^6$ HL60 cells treated with 50μg/ml of C2 for 12 hours with or without prior incubation with RSV (8μM) or catalase (500U/ml). Cyt. C was detected by western blot analysis using a monoclonal anti-Cyt. C IgG, as described in Materials and Methods.

(E) DNA fragmentation was determined by PI staining and the appearance of sub-G1 fraction. $1x10^6$ cells were treated as in B, permeablized and stained with PI as described in Materials and Methods. Cells were analyzed by flow cytometry (10,000 events) and percentage of cells in the various phases of the cell cycle are shown.

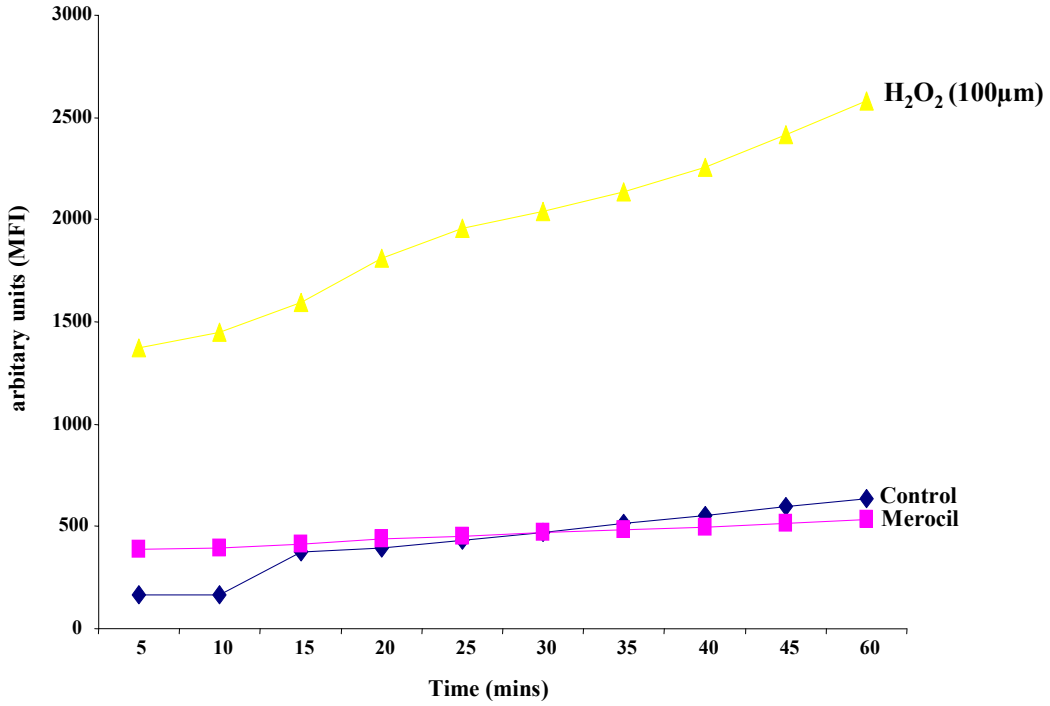


Figure-41: C2 does not produce H_2O_2 in growing medium.

Growing medium (without cells) RPMI was subjected to 100ug/ml of C2 incubated in 6-well plates and loaded with DCF-DA. The samples were incubated in the spectrofluorometer at 37°C from 0-60 minutes. 100uM exogenous H_2O_2 was used as a positive control. Data is represented as MFI against Time.

4.3.6 RSV creates non-permissive environment in inhibition of C2 induced apoptosis:

I next questioned whether the inhibitory effect of RSV on C2-induced apoptosis was also mediated by its ability to create a pro-oxidant intracellular milieu and inhibit cytosolic acidification, two factors that inhibit the execution of the apoptotic signal. Indeed, similar to the results obtained with H_2O_2, pre-incubation of HL60 cells with RSV for 2 hours resulted in an increase in intracellular O_2^- concentration, which was even more pronounced upon subsequent addition of C2 **(Fig-42B**). More importantly, RSV almost completely blocked the drop in cytosolic pH triggered upon exposure to C2 **(Fig-42C)**. Furthermore, pharmacological inhibition of the NADPH oxidase complex by DPI prior to the addition of RSV and C2 restored death signaling in response to **C2 (Fig-42D)**. Indeed H_2O_2 production generated by C2 was completely blocked by catalase on DCF-DA analysis **(Fig-42A)**.

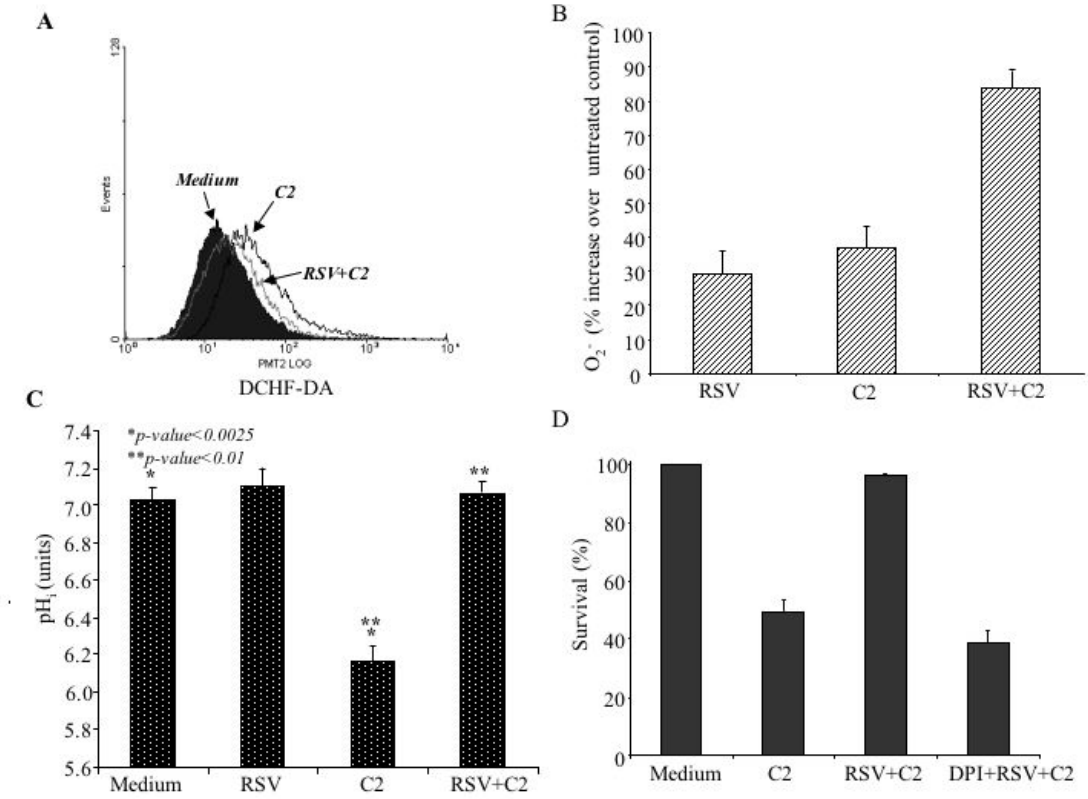

A
Medium
C2
RSV+C2
Events
PMT2 LOG
DCHF-DA
B
O_2^- (% increase over untreated control)
RSV
C2
RSV+C2
C
*p-value<0.0025
**p-value<0.01
pH_i (units)
Medium
RSV
C2
RSV+C2
D
Survival (%)
Medium
C2
RSV+C2
DPI+RSV+C2

Figure-42: RSV creates a pro-oxidant intracellular milieu and inhibits C2-mediated drop in cytosolic pH and cell death.

(A) HL60 ($1x10^6$) cells were treated with 50μg/ml of merocil for 4 hours in the presence or absence of 8μM RSV**.** Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5μM) for 30 min. and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry, as described in Materials and Methods.

(B) $2X10^6$ cells (in 2ml) were treated with RSV (8μM) for 4 hours or 50μg/ml of C2 for 4 hours with or without 2 hours of prior incubation with RSV (8μM) and intracellular O_2^- was measured by a lucigenin-based chemiluminescence assay as described in Materials and Methods. Data are shown as the percentage difference in the intracellular O_2^- concentration compared to untreated control cells and are the Mean S.D of at least three independent experiments.

(C) Cells were treated as in A and cytosolic pH was determined by loading $1x10^6$ cells with the pH sensitive probe BCECF-AM as described in Materials and Methods. pH values were derived from a standard pH curve obtained from cells cultured in different pH buffers in the presence of nigericin (1μg/ml).

(D) HL60 cells ($1x10^6$/ml) were pre-incubated with DPI (1.25μM) or with DPI (1.25μM)+RSV (8μM) for 4 hours prior to the addition of 50μg/ml C2 for 18 hours. Cell survival was assessed by MTT.

4.3.7 Death inhibition by RSV is also implicable to modern anti-cancer drugs-vincristine and daunorubicin:

In order to gain further insight into the death inhibitory effect of RSV and its potential clinical implications, I next investigated the effect of pre-incubation with RSV on apoptosis induced by two chemotherapeutic agents, vincristine and daunorubicin (Pervaiz, 2002). Similar to our results obtained with exogenous H_2O_2 and C2, we show here a dose-dependent effect (up to 8μM) of RSV in significantly reducing the sensitivity of leukemia cells to vincristine **(Fig-43A**). This decrease in sensitivity correlated well with significant inhibition in the enzymatic activities of caspases 9 and 3 **(Fig-43B-C)**. Interestingly, similar to C2, exposure of HL60 cells to vincristine resulted in an intracellular increase in H_2O_2 production, which was significantly inhibited by prior exposure to RSV **(Fig-43C)**. That the inhibitory effect of RSV on death signaling was indeed linked to its ability to create a pro-oxidant intracellular milieu was further supported by the ability of DPI or transient transfection with RacN17 to revert the sensitivity of leukemia cells in the presence of RSV to vincristine-induced apoptosis **(Fig-43D-E).** It is interesting to note that the inhibitory effect of low concentrations of RSV on drug-induced apoptosis was not exclusive to C2 or vincristine. Indeed, pre-incubation of leukemia cells to RSV for 2 hours resulted in a significant increase in cell survival **(Fig-44A**), decreases in caspases 9 and 3 activities **(Fig-44B-C)**, and inhibition of intracellular H_2O_2 production **(Fig-44 D**) upon triggering apoptosis with daunorubicin. These data further corroborate my observations that low concentrations of RSV provide cancer cells with a survival

advantage during drug-induced apoptosis by inhibiting effector components of the death execution program.

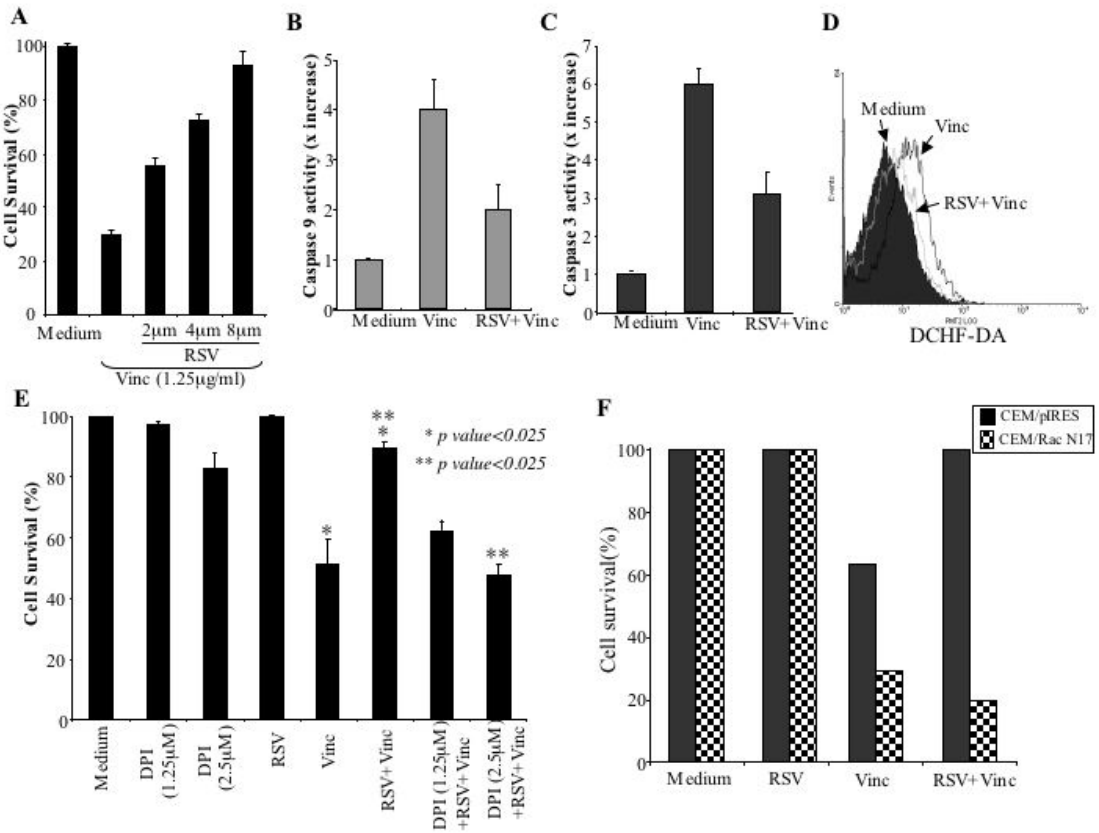

A
Cell Survival (%)
Medium
2μm 4μm 8μm
RSV
Vinc (1.25μg/ml)
B
Caspase 9 activity (x increase)
Medium Vinc RSV+Vinc
C
Caspase 3 activity (x increase)
Medium Vinc RSV+Vinc
D
Medium
Vinc
RSV+Vinc
DCHF-DA
E
Cell Survival (%)
* p value<0.025
** p value<0.025
Medium
DPI (1.25μM)
DPI (2.5μM)
RSV
Vinc
RSV+Vinc
DPI (1.25μM) +RSV+Vinc
DPI (2.5μM) +RSV+Vinc
F
CEM/pIRES
CEM/Rac N17
Cell survival(%)
Medium
RSV
Vinc
RSV+Vinc

Figure-43: The inhibitory effect of RSV on vincristine-induced apoptosis can be neutralized by inhibition of the NADPH oxidase complex.

(A) $1x10^6$ cell/ml were incubated with 1.25μg/ml of vincristine for 18 hours or with RSV (2, 4, or 8 μM) for 2 hours followed by similar exposure to vincristine. Cell viability was determined by the MTT assay as described in Materials and Methods. Data are shown as Mean S.D. of at least three independent experiments performed in triplicates.

(B) (C) Caspase 9 and 3 were described by fluorimetric assays as described before.

(D) HL60 ($1x10^6$) cells were treated with 1.25μg/ml of vincristine for 4 hours in the presence or absence of 8μM RSV**.** Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5μM) for 30 min. and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry, as described in Materials and Methods.

(E) HL60 cells ($1x10^6$/ml) were pre-incubated with DPI (1.25μM or 2.5μM) or with DPI (1.25μM or 2.5μM)+RSV (8μM) for 4 hours prior to the addition of 1.25μg/ml of vincristine for 18 hours. Cell survival was assessed by the MTT assay as described in Materials and Methods. Mean S.D. of three independent experiments is shown.

(F) CEM leukemia cells ($1x10^6$/ml) were co-transfected with a β-gal containing vector and either the empty pIRES vector or the pIRES RacN17 vector. Forty-eight hours post transfection, cells were exposed to 1.25μg/ml of vincrsitine for 18 hours with or without prior incubation for 2 hours with 8μM RSV. Cell survival was assessed by the β-gal survival assay described previously

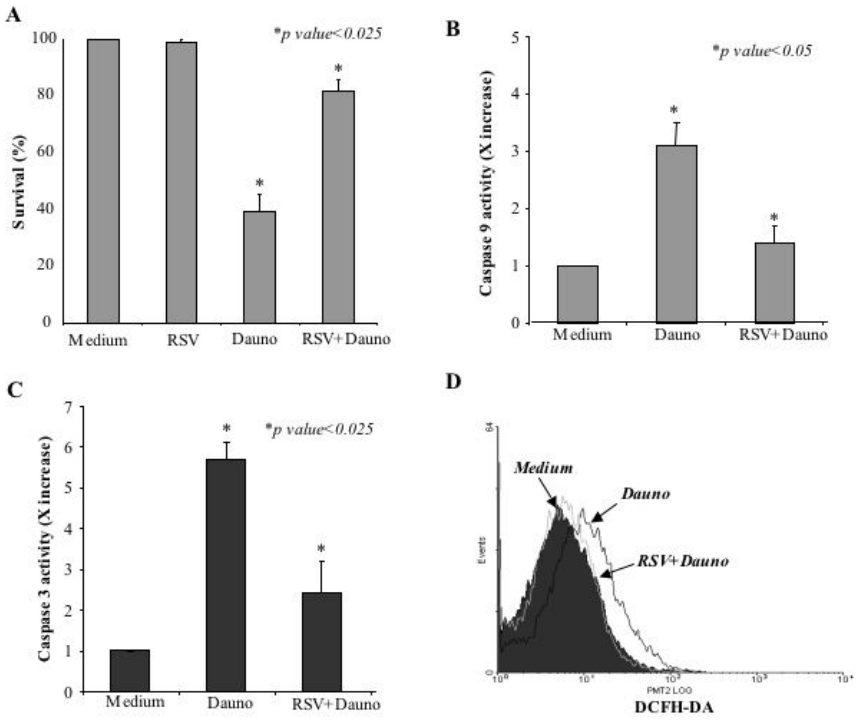


Figure-44: RSV inhibits daunorubicin-induced apoptosis and H_2O_2 production.

(A) $1x10^6$ cell/ml were incubated with 0.2ug/ml of daunorubicin with or without RSV (8uM) for 2 hours. Cell viability was determined by the MTT assay. Data are shown as Mean S.D. of at least three independent experiments performed in triplicates. Lysates obtained from $2x10^6$ cells following exposure to daunorubicin (0.2ug/ml) for 12 hours with or without prior incubation with 8uM RSV were assayed for

(B) caspase 9 and

(C) caspase 3 activities using fluorimetric assays as fold increase (X increase) in enzymatic activity over that of untreated cells' lysates (D) HL60 ($1x10^6$) cells were treated with 0.2ug/ml of daunorubicin for 4 hours in the presence or absence of 8uM RSV.

(D) Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5uM) for 30 min. and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry. Data are shown as Mean S.D. of at least three independent experiments

4.3.8 Decrease in intracellular O_2^- overrides the inhibitory effect of RSV on drug-induced H_2O_2 production:

Thus far I have shown that the inhibitory activity of RSV on drug-induced apoptosis was linked to its ability to increase NADPH oxidase-dependent intracellular O_2^- production. Interestingly, all drugs used in this study resulted in an intracellular increase in H_2O_2 production depicted by the rightward shift in histograms on DCF-DA analysis **(Fig-45)**. More importantly, I investigated the mechanism by which a decrease in intracellular O_2^- induced by incubation with the NADPH oxidase inhibitor DPI restored the sensitivity of cells to drug-induced apoptosis. Here I show that decreasing intracellular O_2^- with DPI virtually completely restored the ability of C2, vincristine, and daunorubicin to trigger intracellular H_2O_2 production even in the presence of RSV **(Fig-45)**. These results establish mitochondrial H_2O_2 production not only as a critical effector mechanism during drug-induced apoptosis but also demonstrate the ability of an increase in intracellular O_2^- to inhibit H_2O_2 production and thereby impeding the recruitment of the mitochondrial death pathway. This lends credence to our hypothesis that intracellular increase in H_2O_2 may be one critical effector mechanism during drug-induced apoptosis, and that factors that negatively influence the intracellular generation of H_2O_2 may render cells non-responsive to chemotherapy (Pervaiz and Clement, 2002b).

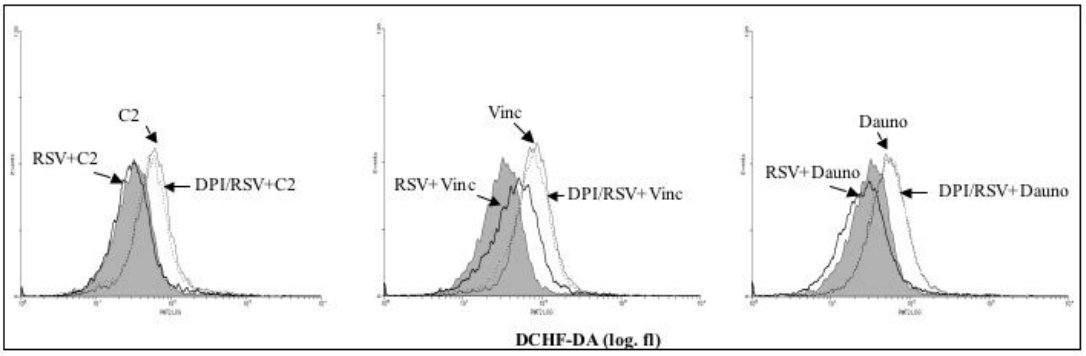


Figure-45: Pre-incubation with DPI reverts the effect of RSV on drug-induced H_2O_2 production.

HL60 ($1x10^6$) cells were treated with of C2 (50μg/ml) or daunorubicin (0.2μg/ml) or vincristine (1.25μg/ml) for 4 hours in the presence or absence of 8μM RSV. In a separate set of experiments, cells were pre-incubated with 1.25μM DPI prior to the addition of RSV (DPI/RSV) and anti-cancer drugs. Cells were then loaded with the H_2O_2-sensitive probe DCFH-DA (5μM) for 30 min. and intracellular H_2O_2 was determined by the shift in fluorescence detected by flow cytometry, as described in Materials and Methods.

4.3.9 RSV inhibits H_2O_2 induced Bax translocation in HL-60 cells revealing that intracellular milieu is critical for death execution:

To correlate and consolidate my previous findings on H_2O_2 and Bax translocation I again proved with the following experiment that intracellular acidification could indeed be a signal for Bax translocation to the mitochondria. The ability of catalase to block H_2O_2 induced acidification by exogenous addition of H_2O_2 or with C1 was first demonstrated to show that intra-cellular acidification is downstream of H_2O_2 production **(Fig-46A-B)**. Similarly, in this experiment exposure to low doses of RSV (4-8uM) for 2 hours and then subsequent addition of H_2O_2 or C1 resulted in significant block in intra-cellular acidification eventually leading to inhibition of mitochondrial translocation of Bax **(Fig-46C-D)**. These experiments provided a link that H_2O_2 mediated death could certainly be impeded by agents such as RSV that block cytosolic acidification and promote survival rather than death of cancer cells.

Short summary of my findings:

I have made an effort to show that drugs can lead to generation to H_2O_2 leading to a drop in pH. The drop in pH recruits Bax to the mitochondria from where cytochrome C is released to engage a cascade of caspases leading to efficient apoptosis. Low doses of RSV can block H_2O_2 mediated signaling. In summary, RSV creates a pro-oxidant state that weakens the mitochondrial circuitry by preventing Bax translocation. It is worthwhile to note that source of $O_2^{\cdot-}$ responsible for endowing a pro-oxidant state was the NADPH oxidase system on the cell membrane. Indeed combination chemotherapy of low doses of RSV blocked the cytotoxic effect of modern chemotherapy drugs vincristine and daunorubicin.

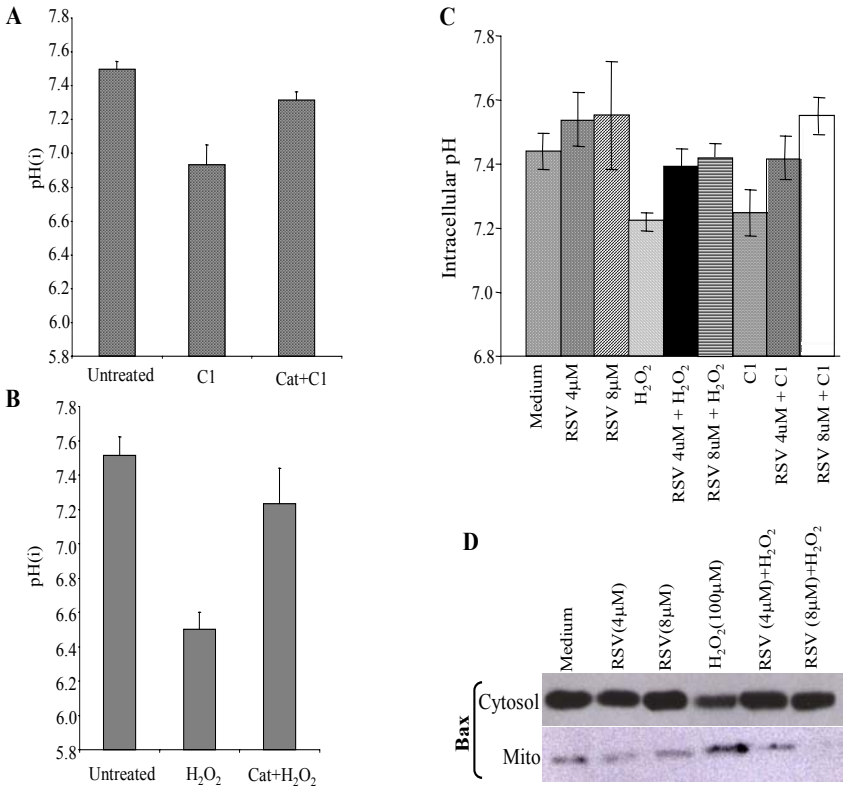


Figure-46: H_2O_2-induced Bax translocation is dependent on H_2O_2-mediated intracellular acidification.

HL60 cells (1x106) were exposed to
(A) 50ug/ml or C1 or **(B)** 100uM H_2O_2 for 6 hours in the presence or absence of 1000U/ml of catalase and intracellular pH was measured with BCECF-AM as described in Materials and Methods.

(C) Cells were pre-incubated for 2 hours with RSV (4 and 8μM) followed by exposure to C1 and H_2O_2 for 6 hours and cytosolic pH was determined as described above.

(D) Mitochondrial and cytosolic fractions from HL60 cells ($2.5x10^7$) following exposure to 50μg/ml of C1 or 100uM H_2O_2 for 12 hours with or without pre-incubation with RSV (4-8μM) were subjected to Western blot analysis using anti-Bax.

5. DISCUSSION

5.1 Role of vitamin C in photo-oxidation of MC540:

Vitamin C by itself has been proven as an apoptosis inducing agent on a variety of tumors (Kang et al., 2003). Data on the use of vitamin C in combination with other drugs has contradictory reports. Recently, a report was published demonstrating that vitamin C upto a dose of 1mM can act as an anti-oxidant when used in combination with drugs like campothecin and flavone, thereby interfering with their apoptosis inducing ability. This report supports previous data on vitamin C to be used as an anti-oxidant (Wenzel et al., 2004). On the other hand, reports have been published to prove that vitamin C could enhance the sensitivity to arsenic mediated cell death in refractory cells like multiple myeloma (Grad et al., 2001).

Our group was interested in deciphering the effect of vitamin C on MC540 mediated cell death under PDT. In this thesis I have highlighted the potential role of vitamin C (that is non toxic alone) but yet have a vital role when photo-activated together with MC540. Indeed MC540 alone has been shown to induce apoptosis in a variety of tumor cell lines such as HT 29, SKco-1 and COLO205 (Chen and Lin, 1996). My initial experiments revealed an increase in tumor cell response to PDT by using a combination of MC540 and vitamin C. This form of therapy has long been used in the treatment of solid tumors (Sharma et al., 2002) (Pass and Pogrebniak, 1992) . However, the experiments clearly indicated that mode of cell death was necrotic in this scenario, although vitamin C significantly potentiated the toxicity of MC540. MC540 is an expensive dye and treatments under PDT are fairly costly. Henceforth,

the advantages lies in using lower doses of MC540 in combination with vitamin C and provide a cost-effective approach of using PDT in clinics as vitamin C is cheaply available in the market. In 1990, a novel approach was designed that would require MC540 to be photo-activated initially followed by the use of its novel photo-products as apoptosis inducing agents without further dependence on light (*pre-activation*) (Pervaiz et al., 1992; Pervaiz et al., 1999b). Using this technique I activated MC540 in the presence of vitamin C, and found that vitamin C speeds up the photo-degradation of MC540 to activated MC540 {p(MC540+VitC)} as shown by the spectro-photograms. Interestingly, I observed virtually complete disappearance of vitamin C during the photo-degradation process. This suggested that vitamin C enhanced the photo-oxidation process of MC540 without interfering in its reaction. and retained its photo-chemical properties. It would have been worthwhile to measure the concentration of ascorbate radical (AH-) but such detection would require great technology and expertise. Another important factor is the fact that AH- radical is short lived and is extremely difficult to measure using techniques like HPLC. Some experiments were tried using the Electronic Paramagnetic Resonance Imaging, but since the apparatus was devoid of having a light source, results were non-conclusive. One possible explanation for increase in photo-degradation of MC540 could be that vitamin C aggregates the photo-sensitizer that facilitates its photo-oxidation and its photo- degradation rate. In this study, it was noticed that vitamin C also enhances the yield of break down products of photo-exposed MC540 after *pre-activation* and thus leads to a generation of an even powerful mixture p(MC540+VitC) that signals via H_2O_2, the most stable of the reactive oxygen species. Inhibition of apoptosis by

catalase/Euk-8 and mannitol suggested the importance of H_2O_2 in these reactions. It is interesting to note that addition of vitamin C on leukemia cells from outside in combination with pMC540540 {*(pMC540+VitC)*} or addition of a photo-exposed mixture of vitamin C {*(pMC540+pVitC)*} had no effect. Only the activation of MC540 in the presence of vitamin C {p(MC540+VitC)} resulted in the most biologically active products. The novel compound derived from these experiments have even better apoptosis inducing ability then compounds derived from pre-activated MC540 alone.

I was intrigued by these findings that vitamin C was completely absent in the new compound p(MC540+VitC) but yet increased its cytotoxic potential. p(MC540+VitC) induced apoptosis in leukemia cells and clearly the addition of vitamin C enhanced the yield of MC540. These findings have provided insight into a positive role of vitamin C on chromphores and addition of vitamin C to other chromphores such as hypericin would be worth investigating.

5.2 Compounds derived from photo-oxidation of MC540 have significant biological activity:

Recently, it was reported MC540 to localizes to the plasma membrane and mitochondria of tumor cells in murine JCS cells (Chen et al., 2000). The importance of photo-oxidation of MC540 is the fact that its degradation yields bio-active compounds that have potential for use anti-cancer agents. The aim to investigate for

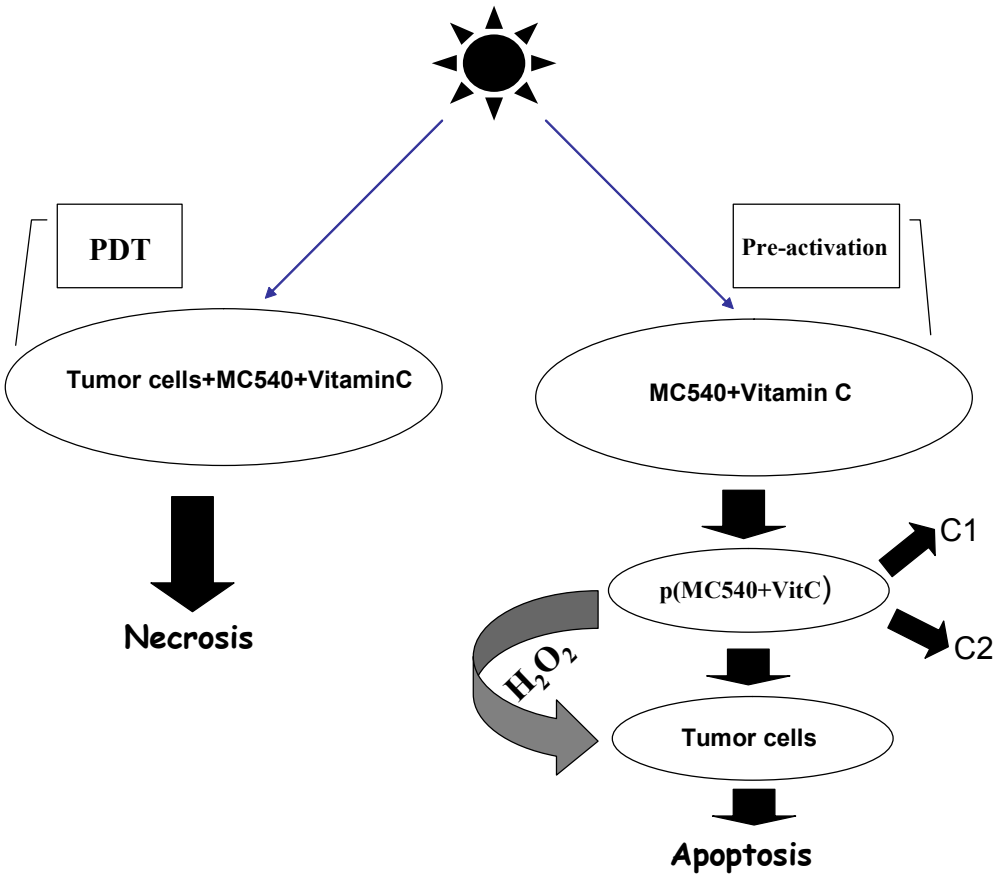
compounds derived from MC540 is that fact these photo-product(s) are of small molecular size, their ability to enter the cells easily and induce effective apoptosis in tumor cells. The compounds C1 (merodantoin) and merocil (C2) (Hirpara et al., 2000) trigger classical hallmarks apoptosis in tumor cells as shown by DNA fragmentation, caspase activation, Bax translocation, drop in mitochondrial transmembrane potential, cytosolic translocation of cytochrome C, formation of apoptotic bodies and cleavage of PARP (Brenner and Kroemer, 2000; Ravagnan et al., 2002). Work started on these compounds since 1994 to show that these novel photo-product(s) have significant anti-tumor activity both in vitro and vivo while demonstrating minimal toxicity to normal cells and tissues (Gulliya et al., 1994a; Gulliya et al., 1994b). However, our group has taken the lead in investigating the ability of these compounds in understanding their mechanism of action(s) and as to whether they have the ability to target the mitochondria, the power generating organelles central to apoptosis. Treatment with these compounds that are less cytotoxic to normal cells will be an ideal scenario, as recently described by the use of compounds like sodium 5,6 benzylidene-L-ascorbate (SBA) (Fujii et al., 2003).

5.3 H_2O_2 - an important mediator in drug induced apoptosis:

Intracellular production of H_2O_2 has been described to be a critical effector mechanism for induction of apoptosis. The production of H_2O_2 within tumor cells changes the environment of the cell such that caspases are more efficiently activated. The remarkable generation of ROS/ H_2O_2 from the agent p(MC540+VitC) suggests

the significance of ROS signaling which can be a measure of the potency for any compound or at least in part a measure of its apoptosis inducing ability. The abrogation of p(MC540+VitC) induced cell death/apoptosis using H_2O_2 scavengers has further substantiated the fact that drugs may directly generate ROS within the cell and blocking such production could shut down the apoptosis machinery that is critical to cell death. These findings will pave way for investigating the effects of vitamin C on other chromophores and understanding the relationship between ROS signaling and cell death. **MODEL 1** is an illustrative summary of of my findings.

These findings have again established the significance of H_2O_2 production and strongly supports our redox hypothesis (Clement and Pervaiz, 2001; Pervaiz and Clement, 2002b). One can enquire as to why drugs acting via H_2O_2 are of such importance. In this context, recently a report was published, that strongly supports our hypothesis. The report discusses that malignant cells are more active than normal cells primarily the fact that they have a high intrinsic oxidative stress and are more vulnerable to damage by ROS producing agents(von Harsdorf et al., 1999). This biological difference between tumor and normal cells would perhaps hold the key to therapeutic selectivity and that designing anti-cancer strategies to preferentially kill tumor cells and being less toxic to normal cells would be a great breakthrough in cancer research (Hileman et al., 2004).



MODEL 1: A model revealing switching of cell death mechanism from necrosis to apoptosis under PDT and pre-activation respectively.

Upon treatment with MC540 and vitamin C, HL-60 cells exhibited classical necrosis under PDT. The increased response of tumor cells to MC540+VitC was exploited by pre-activating the MC540 and vitamin C mixture under light first and then using it as an anti-cancer compound. This mixture p(MC540+VitC) acts via hydrogen peroxide to kill tumor cells by classical apoptosis. In addition, the use of light required simultaneously was also circumvented. Compounds derived from this technique are better on yield then those derived from MC540 alone.

5.4 H_2O_2 and its critical role in cellular proliferation and apoptosis:

The critical role of intracellular or exogenously added H_2O_2 in cellular injury and death is well established and also pertaining to MC540 its toxicity to cells has been previously reported (Bruchelt et al., 1989). Depending upon their intracellular concentration and the state of cellular anti-oxidant defense systems, H_2O_2 can induce cells to undergo necrosis or trigger the cells' apoptotic machinery (Burdon et al., 1996; Clement and Pervaiz, 1999). Consistent with this view, our recent findings have highlighted the regulatory role of intracellular ROS in the apoptotic pathway(Clement and Pervaiz, 2001; Pervaiz et al., 1999a). In addition to providing evidence that exposure of cells to H_2O_2 can induce necrosis or apoptosis (concentration dependent), we also demonstrated that an increase in intracellular H_2O_2 is an effector mechanism during drug-induced apoptosis of tumor cells (Clement et al., 1998b). The latter and many other related studies strongly argue in favor of a signaling role for ROS such as H_2O_2 during apoptotic commitment or execution(Burdon, 1995; Burdon, 1996; Burdon et al., 1996; Burdon et al., 1989). To that end, low concentrations of H_2O_2 have been implicated in the activation of transcription factors, such as NF-kB, and up regulation of mRNA expression of p53, COX-2, and the Bcl-2 family member Bax in some systems (Nakamura and Sakamoto, 2001; Simizu et al., 1998).

Exogenously hydrogen peroxide has been shown to induce apoptosis in variety of tumor models and considerable data has been published on the apoptosis inducing ability of this agent (Chang et al., 2003; Jiang et al., 2003). Moreover, data is

emerging on agents such as glutathione that are been shown to signal directly via hydrogen peroxide production (Perego et al., 2000)

ROS have been strongly connected to Bcl-2 family members as localization of these proteins in nucleus, mitochondria and ER are all ROS production sites (Korsmeyer et al., 1995). Relationship between oxidative/reductive stress and major pro-apoptotic molecules like Bax is still confusing. Because H_2O_2 was strongly implicated in our reactions and our novel compounds directly induced intracellular H_2O_2 production we wanted to investigate one step further to see whether exogenous addition of H_2O_2 or using novel compound C1 engages the mitochondria by involving the most important pro-apoptotic protein Bax. Prior to this study C1 had never been used and studied in context to mitochondria. The object of using C1 was to provide information on its mechanism of action and data generated on this compound would be essential in understanding signaling pathways mediated by H_2O_2. Indeed , I have clearly shown that C1 is responsible for intracellular H_2O_2 production and its killing effect is H_2O_2 dependent.

5.5 Engagement of the mitochondrial circuitry in H_2O_2 mediated apoptosis:

The involvement of the mitochondrial death circuitry during H_2O_2-induced apoptosis has been well documented (Dumont et al., 1999; Hirpara et al., 2001; Li et al., 2003; Stridh et al., 1998; Takeyama et al., 2002). Not only does the mitochondria serve as a source for intracellular ROS, in particular H_2O_2 (Cadenas and Davies, 2000), but they are also a target for attack by ROS leading to the release of death amplification

factors (Madesh and Hajnoczky, 2001; Polla et al., 1996). This is also evidenced by the ability of Bcl-2 to inhibit apoptosis induced by H_2O_2 (Bruce-Keller et al., 1998; Clement et al., 2003; Dumont et al., 1999). The key factor was to investigate the relationship between H_2O_2 and Bax. This model has been highlighted for organisms such as yeast where apoptosis can be induced by low doses of H_2O_2 or by expression of mammalian Bax. Indeed, radical depletion or their scavenging results in the prevention of yeast undergoing apoptosis (Madeo et al., 1999)

Aside from the up regulation of the pro-apoptotic protein Bax upon exposure of some cell types to H_2O_2, the inhibitory effect of over expression of the anti-apoptotic protein Bcl-2 on H_2O_2-induced apoptosis(Esposti et al., 1999), supports the involvement of the mitochondrial death pathway in apoptotic signaling by H_2O_2. However, as the mitochondria are a major source of intracellular ROS production, it is tempting to speculate that ROS such as H_2O_2 may function both upstream and downstream of the mitochondria. If so, the upstream function could be the recruitment of the mitochondrial pathway via translocation of Bax.

Pro-apoptotic members of the Bcl-2 family, such as Bax, Bid, Bad, and others undergo conformational change upon triggering of apoptosis(Desagher et al., 1999; Gross et al., 1998; Simonen et al., 1997). Bax and Bid are cytosolic proteins that can translocate to the mitochondria and form homo- or heterodimers with other Bcl-2 family members. Insertion of Bax in the mitochondrial outer membrane results in the formation of channels that could permit the release of inter-membrane proteins such as cytochrome C (De Giorgi et al., 2002; Marzo et al., 1998). It has been recently

reported that cells lacking Bax (Bax-/-) are resistant to the effect of some anti-cancer drugs (Zhang et al., 2000).

5.6 H_2O_2 is a signal for Bax translocation to the mitochondria:

In agreement with that, we show here that HCT116 Bax (-/-) cells are completely resistant to death induced by apoptotic concentrations of H_2O_2, unlike Bax+/- cells. Furthermore transient transfection of Bax in HCT116 Bax(-/-) cells restored their sensitivity to H_2O_2, thus establishing the critical role of Bax in H_2O_2-induced apoptosis. One probable mechanism underlying the differential sensitivity of Bax+/- and Bax-/- cells to H_2O_2 could be that H_2O_2 increases the expression of Bax or alters the cellular Bax/Bcl-2 ratio in Bax+/- cells as suggested by some reports (Maroto and Perez-Polo, 1997). However, our results show that the Bax/Bcl-2 ratio was not significantly altered upon 4-24 hours exposure to exogenous addition of H_2O_2. This was concomitant to findings observed on other cells such as neuronal and cardiomyocytes where Bcl-2 expression was unaltered on exposure to H_2O_2 (Kitamura et al., 1999). However, in one of the studies ROS mediated hypertrophic changes in cardiac muscle have been described to induce apoptosis in response to H_2O_2 and were directly linked to Bax expression (Kitamura et al., 1999; Pimentel et al., 2001).

Furthermore, some reports have shown that although Bcl-2 and Bax expression can be altered upon H_2O_2 exposure, in a dose dependent manner but this parameter cannot be used to define whether a cell will undergo or survive apoptosis (Maroto and Perez-Polo, 1997). Conformational change like Bax cleavage have been described in some cells such as hepatocytes by H_2O_2 treatment but this phenomenon was not observed in

any of our experiments either by exogenous addition of H_2O_2 or C1 (Tamura et al., 2003).

Interestingly, analysis of sub-cellular distribution of Bax (in HCT116, HL60 and CEM cells) revealed that Bax redistributed to the mitochondrial fraction from the cytosol upon exposure to H_2O_2, which could be significantly blocked by the H_2O_2 scavenger catalase and perhaps is the most important aspect of H_2O_2 induced mitochondrial apoptosis. In support of my experiments very recently findings on hyperoxia induced Bax activation have been published. Exposure of hyperoxia in alveolar epithelial cells resulted in activation of Bax at the mitochondrial membrane subsequently leading to cyt C release. These events were completely blocked by pre-incubation with catalase mimetic EUK-134 (Buccellato et al., 2003).

One could always argue that the changes elicited upon exposure of cells to exogenous H_2O_2 may not have physiological relevance or practical implications (although H_2O_2 is being used as an unconventional treatment on tumor cells), as such high levels of intracellular H_2O_2 are rarely observed in living cells. Therefore, in order to present a more real life situation, I exploited the ability of certain anti-cancer drugs to increase intracellular production of ROS, specifically H_2O_2. In this regard, we have recently shown that a novel anti-cancer drug merocil triggers H_2O_2-dependent apoptosis in human tumor cells (Hirpara et al., 2001). In this study, another novel anti-cancer compound C1 (merodantoin), the apoptosis inducing activity of which has recently been reported (Pervaiz et al., 1999c), was used as a trigger for apoptosis. Exposure of HCT116 Bax +/- or HL60 cells to C1 resulted in an increase in intracellular H_2O_2 (as

early as 4 hours) and translocation of Bax to the mitochondria. This translocation of Bax was completely inhibited by the H_2O_2 scavenger catalase, thus establishing the critical role of intracellular H_2O_2 in drug-induced apoptosis of tumor cells.

5.7 H_2O_2-dependent Bax translocation is caspase independent and does not involve CD95 pathway:

Recruitment of Bax to the mitochondria during apoptotic signaling has been linked to the activation of upstream caspase 8 and caspase 8-mediated cleavage of the pro-apoptotic protein Bid (Desagher et al., 1999). This is particularly true upon ligation of death receptors, such as CD95 (Apo1/Fas). Incidentally, H_2O_2 and anti-cancer drugs have been shown to up regulate the expression of the CD95 receptor or its ligand (CD95L) in some systems. In these systems, blocking receptor signaling or downstream caspase activation abrogates Bax translocation and consequently the death signaling circuitry downstream of the mitochondria. Interestingly, in our study neither of the stimuli triggered up regulation of the CD95 receptor, and inhibition of caspase activation by pan-caspase inhibitor z-vad had no effect on the mitochondrial translocation of Bax triggered by C1 or exogenously added H_2O_2. Prior to these findings it has been shown that H_2O_2 induced apoptosis is CD95 independent and that it primarily requires the release of mitochondrial derived ROS together with NF-kB activation (Dumont et al., 1999). Similar findings were observed with generation of ROS and these are discussed below. In addition, the relative lack of caspase 8 activation and absence of downstream Bid cleavage provide further evidence in favor of a mechanism for signaling Bax from the cytosol to the mitochondria that is either

parallel to or independent of caspase activation. A similar mechanism of caspase independent conformational change of Bax upon triggering apoptosis has recently been reported (Bellosillo et al., 2002).

5.8 Ceramide production does not play a role in H_2O_2 dependent Bax translocation:

Recent findings also implicate ceramide production in response to activation of the membrane sphingomyelinase in signaling Bax to the mitochondria (Sawada et al., 2000; von Haefen et al., 2002). Ceramide production is observed in a variety of apoptotic models, including receptor and drug-induced apoptosis (Delpy et al., 1999; Sawada et al., 2000). To that end, H_2O_2 has been shown to trigger intracellular ceramide production in tumor cells (Goldkorn et al., 1998; Mansat-de Mas et al., 1999). Indeed, exposure of HL-60 cells to H_2O_2 or to C1 resulted in significant production of ceramide. Intrigued by these findings, I questioned whether ceramide production could be the stimulus downstream of H_2O_2 for mitochondrial localization of Bax. I exploited the inhibition of sphingomyelinase activity by gentamycin sulfate to evaluate the effect of ceramide on C1-induced Bax translocation. Inhibiting ceramide production in this system did not affect the sub-cellular localization of Bax triggered by drug exposure or upon exogenous addition of H_2O_2. Collectively, these data indicate that Bax translocation triggered in tumor cells during drug (C1)-induced apoptosis may be a direct result of intracellular H_2O_2 production, independent of the upstream caspase 8 or ceramide pathways. This findings were concomitant with reports that demonstrate that H_2O_2 induced caspase 3 and 9 activation was independent of ceramide production and that ceramide was not required for H_2O_2

killing (Yamakawa et al., 2000). Corroborating these findings is also a recent report suggesting glutathione-dependent activation of Bax by oxidative stress in HeLa cells stably transfected with the cystic fibrosis transmembrane conductance regulator (Jungas et al., 2002).

5.9 Drug-induced Bax translocation is dependent upon H_2O_2 mediated cytosolic acidification:

pH regulation both intra and extra-cellularly has been shown to have an important role in tumor development. Cellular pH has been shown to be critical for cell proliferation, invasion and metastasis, drug resistance and most importantly apoptosis (Izumi et al., 2003). Understanding the mechanism(s) involved in pH regulation of cancer cells will provide new ways of inducing cancer specific apoptosis that can aid cancer therapy.

We have recently demonstrated that intracellular H_2O_2 production is a critical effector mechanism during drug-induced apoptosis of tumor cells. In this regard, exposure of cells to H_2O_2 or to drugs that trigger intracellular increase in H_2O_2results in a significant drop in cytosolic pH. Cytosolic acidification is an early event in apoptosis described by us and many others and provides an intracellular environment permissive for efficient death execution. Accordingly, signals that inhibit apoptotic acidification impede death signaling as demonstrated in our recent communication (Pervaiz and Clement, 2002b). My results provide strong evidence that the link between H_2O_2 and Bax translocation could be the drop in cytosolic pH brought about by exposure of cells to exogenous H_2O_2 or endogenous production of H_2O_2 upon drug

exposure. Not only did inhibition of cytosolic pH drop triggered by H_2O_2 or C1 result in a significant reduction in mitochondrial localization of Bax, but most interestingly clamping cytosolic pH to a more acidic range (around 7.1) directly induced translocation of Bax in tumor cells. Amiloride, has been used for classical targeted inhibition of NHE-1 and thus blocking the efflux of H^+ ions in lieu of Na^+ ions would render the cells to an acidic environment. Researchers have documented its ability to induce cytosolic acidification (Coakley et al., 2002; Marches et al., 2001). As caspase inhibitors had no effect in our system, these results point to a direct effect of an acidic intracellular milieu in trafficking Bax from the cytosol to the mitochondria. It is interesting to note that one of the first papers to report a relationship between cytosolic pH and Bax translocation had concluded to the contrary, i.e. pH increase was shown to facilitate Bax translocation (Khaled et al., 1999). However, in this system there was absence of any initial increase in cytosolic pH downstream of H_2O_2 or C1 even as early as 30 minutes post-treatment. Interestingly, in our system H_2O_2 production could be detected early to cytosolic acidification, substantiating the fact that pH drop is downstream of H_2O_2 and is required for charging of apoptotic machinery like caspases. Indeed it has been demonstrated that activation of cytosolic caspases by cytochrome C was maximal at acidic pH and a neutral pH dampens the effectivity of these enzymes. Furthermore, it has been shown that in yeast cells "ectopic expression of Bax in wild type, but not FoF1/H+ pump deficient cell results in mitochondrial matrix alkanization with reciprocal cytosolic acidification and eventually cell death (Matsuyama et al., 2000). Not only in mitochondrial dependent apoptosis but recently reports have emerged revealing that decrease in pH could

augment TRAIL induced apoptosis and thus low pH environments would enhance receptor mediated cytoxicity (Lee et al., 2004) or drop in pH by drugs such as staurosporine could result in Bax activation consequently leading to apoptosis (Tafani et al., 2002). In my experiments inhibition of pH drop by agents like RSV abrogated Bax translocation, and the direct effect of acidic intracellular milieu by treatment with amiloride on Bax translocation, provide strong evidence that acidic pH is a direct signal for Bax translocation even in the absence of any stimulus. Although, all the cell lines are p53 proficient, as a future line of investigation it would be interesting to explore whether RSV induced inhibition is p53 dependent or independent since p53 is an upstream signal for Bax and is central to defects in cancer cells that results in their abnormal proliferation.

5.10 H_2O_2-mitochondrial Bax- H_2O_2: ROS-dependent ROS production:

The results also suggest a novel role for ROS, such as H_2O_2 in the engagement of the mitochondrial death machinery during drug-induced apoptosis. Earlier reports have implicated O_2^- and H_2O_2 as probable species responsible for mitochondrial damage, such as lipid peroxidation of the mitochondrial membranes especially described in yeast models (Priault et al., 2002) and induction of MPT (Asumendi et al., 2002), changes that could explain for the increase in the permeability of the outer membrane and the leakage of pro-apoptotic proteins to the cytosol. Using yeast as a model it has been demonstrated that expression of Bax in yeast cells generated ROS and Bax inhibiting genes like ascorbate peroxidase (sAPX) when co-expressed greatly reduce this ROS generation, which in turn suppresses Bax induced cell death in yeast (Moon

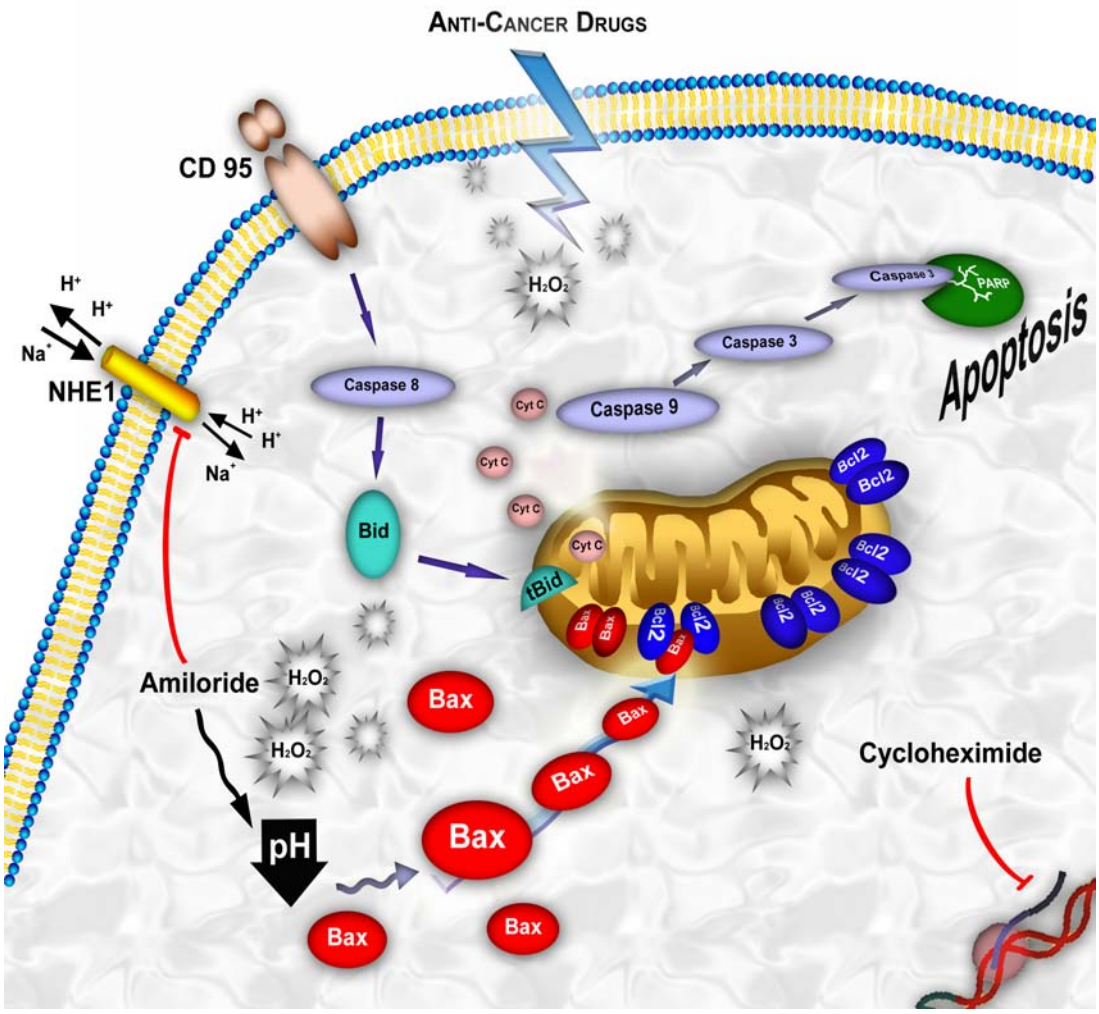
et al., 2002). However, most of these studies seem to imply that the mitochondrial burst of H_2O_2 is likely to be a downstream effector mechanism for the execution signal. My data argues strongly in favor of a "two-hit" function for intracellular H_2O_2. The initial increase (first hit) in intracellular H_2O_2 upon drug treatment of tumor cells leads to the targeting of Bax to the mitochondria in a caspase-independent manner, thus resulting in the recruitment of the mitochondrial death pathway. Insertion of Bax into the mitochondrial outer membrane directly or interacting with mitochondrial membrane proteins like VDAC could then bring about mitochondrial changes, such as induction of MPT, formation of a functional channel in the outer membrane, and as our data suggest serve as a stimulus for the next burst of H_2O_2 (second hit) from the mitochondria. This could lead to peroxidative damage of mitochondrial lipids, such as cardiolipin, and facilitate the egress of Cyt.C, Smac/Diablo, or AIF. This phenomenon of ROS-induced ROS production has been described before as a probable mechanism for the induction of MPT, and more recently in a model of nerve growth factor-deprived neuronal cell death Bax insertion has been proposed to induce ROS burst, strong enough to trigger the release of Cyt.C(Kirkland et al., 2002). One probable mechanism for Bax-induced H_2O_2 production could be that Bax translocation leads to Cyt.C release which depletes the mitochondrial electron transport chain with the resultant leakage of electrons to molecular oxygen to form O_2^-, which is then dismutated by the mitochondrial superoxide dismutase (MnSOD) to H_2O_2.

The next set of experiments would be to closely look at co-factors such as calcium. Mechanisms where neuronal cells generate ROS in the presence of calcium and Bax

together have been recently described implying that calcium can be one of the key players in translocation of Bax (Starkov et al., 2002). Using the Bax inhibitor 1 (BL-1) that has been shown to suppress Bax mediated cell death in yeast and Arabidopsis in response to triggers like H_2O_2 and can been used in mammalian tumor cells to see whether this can inhibit ROS generation (Kawai-Yamada et al., 2004). The most important question to be addressed would to be to investigate the effect of acidic pH on Bax movement. Does decrease in pH oxidize certain sequences in the Bax protein that would be essential for its translocation? In this respect it would be also interesting to look at vesicle associated membrane protein (VAMP). Recently it has been shown that Bax induced apoptosis was suppressed by transformation with Arabidopsis VAMP and that this AtVAMP blocked Bax induced cell death downstream of oxidative burst. Studying whether decrease in pH has association wit VAMPs would provide in depth insight to translocation of Bax in an acidic environment (Levine et al., 2001).

The work presented here consolidates earlier findings from our group that intracellular H_2O_2 is an effector mechanism during drug-induced apoptosis of tumor cells and provides a mechanistic explanation for its ability to connect extra-mitochondrial death circuitry to the mitochondria, the point of convergence for effective drug-induced death signals.

MODEL 2 is an illustrative summary of my findings on how a reductive environment created by H_2O_2 could engage the mitochondria using Bax via drop in pH (cytosolic acidification),



MODEL-2: A proposed concept in hydrogen peroxide signaling.

Hydrogen peroxide induced cytosolic acidification is critical for Bax translocation. This translocation is independent of receptor signaling, caspase activation and does not require new protein synthesis. However, intracellular acidification is critical in H_2O_2 such that artificially clamping the pH using an NHE-1 inhibitor like amiloride enforces Bax to the mitochondria resulting in cytochrome C release that recruits the caspase cascade imperative for degradation of cell repair enzymes such as PARP.

5.11 Switching the death signal to survival: testing the redox hypothesis

So far I have discussed that tumor cell response to drug induced apoptosis is detrimental by H_2O_2 production and a reductive environment. Ideally, if one could prove the reciprocal practically using drugs in context to the changes in the intracellular redox status. To test our hypothesis and to justify that a critical balance between H_2O_2 and O_2^- practically exists I hereby discuss another model. Using resevratrol at low doses I observed interesting findings that had not been documented before. These experiments highlight yet another interesting property of this remarkable natural product known for its cancer chemopreventive, apoptosis inducing, anti-inflammatory, anti-atherogenic, and immune modulatory properties (Pervaiz, 2003).

Before discussing the findings of RSV on leukemia cells in response to various apoptotic insults including H_2O_2, some interesting observation are worth to point out that have been made in respect to human erythroleukemia K562 cells. Here the group has reported that RSV interferes with the elevation of LB4 and PE2, the main arachidonate metabolizing enzymes and consequently cells resisted the H_2O_2 challenge. Thus its inhibition on inflammatory cells in response to H_2O_2 was somehow established.(MacCarrone et al., 1999; Martinez and Moreno, 2000)

The results in this thesis provide strong evidence that contrary to its pro-apoptotic activity at higher concentrations (25μM and above) low micromolar concentrations could have a protective effect on the cells. More interestingly, we show here that at

such low concentrations *in vitro*, RSV has a potent effect on the intracellular redox status, a critical determinant of the efficacy of the death signal (Clement and Pervaiz, 1999; Fadeel et al., 1998; Hampton and Orrenius, 1998).

Tumor cells possess numerous systems that produce ROS, that can induce apoptosis or necrosis, but at physiological levels moderate levels can modulate transcription and expression of survival genes. "ROS are also found to mimic some of the physiological stimuli by direct modification of factors or indirect mechanisms via change in the oxidative and reductive status inside/outside cells." (Nose, 2000)

In that respect, it has previously been shown that maintaining a slightly elevated intracellular concentration of O_2^- promotes cellular proliferation (Burdon, 1995; Burdon, 1996) and inhibits death signaling via a direct or indirect effect on caspase activation pathways (Fadeel et al., 1998; Pervaiz et al., 1999a). Indeed, a pro-oxidant intracellular milieu is an invariable finding in cancer cells and has been shown to provide them with a survival advantage over their normal counterparts (Cerutti, 1985). In support of this hypothesis, I previously demonstrated that decreasing intracellular O_2^- via pharmacological inhibition of the NADPH oxidase complex or by transfection of tumor cells with the dominant negative form of Rac (RacN17) significantly increased the sensitivity of tumor cells to apoptotic stimuli (Clement and Stamenkovic, 1996; Pervaiz et al., 2001). More recently, our group has linked the ability of Bcl-2 to inhibit death signaling in human leukemia cells to its pro-oxidant property and more importantly showed that decreasing intracellular O_2^- could bypass Bcl-2 protection and restore sensitivity of leukemia cells to apoptotic triggers (Clement et al., 2003). Contrarily, inhibition of superoxide dismutase (Cu/Zn SOD),

a critical enzyme that dismutates O_2^- to generate H_2O_2, resulted in a significant increase in the constitutive intracellular levels of O_2^- and a reciprocal decrease in cell sensitivity to apoptosis (Pervaiz et al., 1999a). Therefore, the ability of H_2O_2 to induce a significant drop in intracellular O_2^- (Clement et al., 1998b) could be a critical effector mechanism for sensitizing tumor cells to death triggers.

5.12 Pro-oxidant state induced by RSV inhibits H_2O_2 induced apoptosis:

Reports are emerging to describe protection by RSV to H_2O_2 induced DNA damage and similarly with agents such as curcumin and tacrine that have been very recently documented (Liu and Zheng, 2002; Wang et al., 2002).

The inhibitory activity of RSV on H_2O_2-mediated death signaling could be attributed to its ability to maintain a pro-oxidant concentration and blocking H_2O_2-induced cytosolic acidification, thereby creating an environment non-permissive for caspase activation and efficient death execution. I was intrigued by the observation that exposure of cells to low concentrations of RSV resulted in an increase, rather than a decrease, in intracellular O_2^- concentration.

Our work over the past few years and recent observations from other groups have established the regulatory role of intracellular O_2^- on apoptotic death signaling (Clement et al., 2003; Clement and Pervaiz, 1999; Fadeel et al., 1998; Lin et al., 1999; Pervaiz et al., 2001; Sen et al., 1999). In this regard, intracellular O_2^- was shown to inhibit death triggered either via ligation of the CD95 (Apo1/Fas) death receptor or by exposure to anti-cancer drugs (Clement and Stamenkovic, 1996;

Pervaiz et al., 1999a). Contrarily, decreasing intracellular O_2^- by inhibition of the NADPH oxidase complex significantly enhanced the sensitivity of tumor cells to apoptotic stimuli (Pervaiz et al., 2001; Pervaiz et al., 1999a). Considering the reports on the anti-oxidant potential of polyphenolic compounds such as RSV (Fang et al., 2002; Jang and Surh, 2001; Kim et al., 2002; Olas and Wachowicz, 2002; Sinha et al., 2002), I observed the pro-oxidant effect prior to its anti-oxidant effect. Indeed RSV blocked cytosolic acidification induced by H_2O_2 or by H_2O_2 producing drugs like C2. Also taking into consideration the earlier reported ability of RSV to inhibit mitochondrial complex III-induced ROS production (Zini et al., 2002), our paradoxical findings provide evidence for a pro-oxidant effect of RSV at concentrations that do not induce cell death or damage. Indeed, such pro-oxidant activity of polyphenolics, such as RSV, has been recently reported in different systems (Martinez et al., 2002; Tinhofer et al., 2001). In one model, ROS generation at concentrations of RSV that triggered cell death in human cancer cells was proposed to be responsible for its cytotoxic activity (Tinhofer et al., 2001).

5.13 RSV hits NAPDPH oxidase system that is responsible for inhibition of H_2O_2 mediated apoptosis:

The results also point to the membrane NADPH oxidase complex as a potential source of O_2^- upon incubation with low doses of RSV, and the pro-oxidant state created by this slight increase as the factor responsible for its death inhibitory activity. These data are in agreement with our recent *findings* demonstrating that human melanoma M14 cells expressing the constitutively active form of Rac (RacV12)

maintained a significantly higher intracellular concentration of O_2^- and were relatively resistance to apoptosis, and contrarily RacN17 expression enhanced sensitivity of M14 melanoma and CEM leukemia cells to death stimuli by decreasing intracellular O_2^-(Clement et al., 2003; Pervaiz et al., 2001). In the light of these data, it is plausible that the inhibitory activity of RSV could be linked to its ability to activate the NADPH oxidase complex, which could explain for the slight increase in intracellular O_2^- upon exposure of cells to low concentrations of RSV. This could then result in a slight pro-oxidant intracellular environmentdoes not promote efficient apoptotic execution (Clement and Stamenkovic, 1996; Pervaiz et al., 1999a).

Not only did inhibition of the NADPH oxidase complex restore death signaling, but also resulted in reverting the negative effect of RSV on drug-induced intracellular H_2O_2 production. By implication, this suggests that an increase in intracellular O_2^- could inhibit downstream H_2O_2 production from the mitochondria thereby inhibiting the drop in intracellular pH, a critical determinant for efficient death execution. It is indeed intriguing as to how an increase in intracellular O_2^- signals to block mitochondrial-derived H_2O_2. One possibility could be inhibition of upstream caspase activation or impeding pro-apoptotic molecules like Bax that weakens the recruitment of the mitochondrial pathway thereby resulting in minimal H_2O_2 production and downstream Cyt.C release. This in turn fails to amplify the caspase cascade and leads to a substantial decrease in the cells' sensitivity to apoptotic stimuli that require mitochondrial amplification factors.

5.14 Inhibition of apoptosis by low dose RSV is applicable to our novel compounds and modern chemotherapeutic agents:

One can argue the use of H_2O_2 in modern medicine or as a chemotherapeutic agent in anti-cancer therapy. Since the data on C2 had been documented, I set out to use it as drug that acts via H_2O_2. Classically, the experiments were replicated using the low dose inhibition by RSV with C2 as shown for exogenous H_2O_2. In addition, the experiments have indeed shown that this inhibition was not specific to C2 but was also observed with known chemotherapeutic agents vincristine and daunorubicin.

Indeed, these drugs had the capability to produce ROS in particularly H_2O_2. Interesting data is recently emerging to show that part of their apoptosis inducing ability could be attributed to their ROS production or regulation.(Groninger et al., 2002; Tsang et al., 2003) Role of ROS in relation to these compounds holds potential for further investigation.

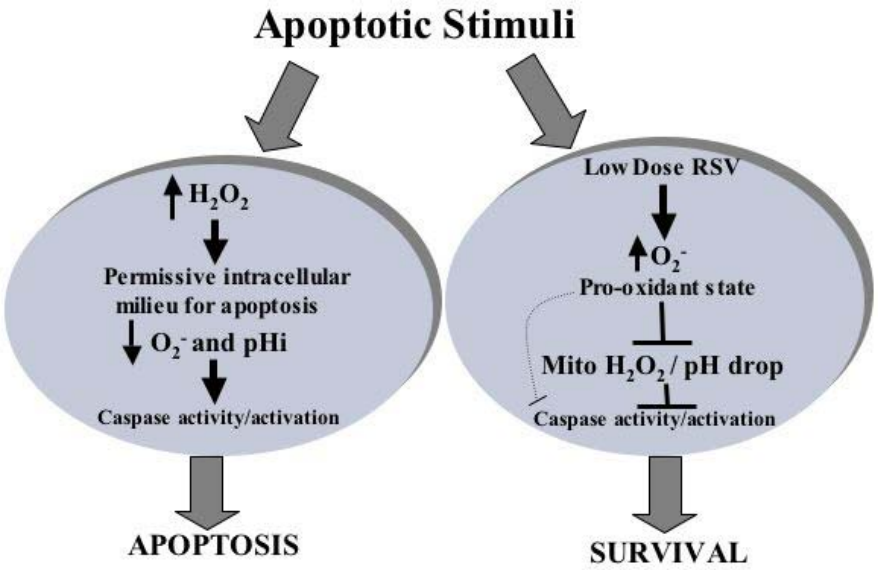
5.15 RSV at low doses: a dangerous cocktail in combined chemotherapy:

These findings could be potentially important in the light of the recent interest in the biological activity of flavanoids or flavanoid-like molecules, such as RSV, for their possible use in combination chemotherapy regimens. Although in vitro exposure of tumor cells to RSV at relatively high concentrations result in apoptotic cell death, plasma levels of RSV as high as 50-100μM may not be physiologically attainable. It

is more likely to reach a sustained plasma level of RSV in the low micromolar range, similar to that shown in this study. Therefore, from the clinical standpoint these data strongly suggest a death inhibitory and/or pro-survival activity of RSV in leukemia cells at doses that may be physiologically relevant. Thus the use of RSV in combination with drugs such as C2, vincristine, or daunorubicin could be a dangerous cocktail as the slight pro-oxidant effect elicited in tumor cells may provide tumor cells with a survival advantage on the one hand and impede death signals on the other. This could then be an ideal environment for the propagation and proliferation of tumor cells. It is to be taken into account that these findings do not contradict the protective effects of RSV on normal cells (Mizutani et al., 2001). However, as a future perspective effect of low doses of RSV on normal primary human cells would be worth investigating. If RSV at low doses facilitates anti-apoptotic signals in normal cells, or contrarily these low doses potentiate killing of normal cells with anti-cancer drugs would be of value and worth investigating. The precise mechanism underlying the ability of an increase in intracellular O_2^- to inhibit mitochondrial H_2O_2 production is the focus of further investigation by our group. Another area of significance would be to look at proteins targeted by RSV at low doses. I have already shown results where RSV at low doses arrests the cells at S phase. As a future line of investigation the role of cyclins would be worth investigating. Recently, it has been shown that Akt activates the NADPH oxidase in vitro by phosphorylating serines S304 and S328 of p47(PHOX) a component of the activated NADPH system. Perhaps RSV could activate Akt that in turn hits the NADPH oxidase system thereby producing O_2^- that is responsible for its inhibitory effect on apoptosis inducing

agents.(Chen et al., 2003; Hoyal et al., 2003) Another important protein of interest to investigate would be anti-apoptotic protein Bcl-2. Bcl-2 over expression has been shown to attenuate RSV induced apoptosis in one model, but here the question would be whether low doses induce increased Bcl-2 expression and generate survival signals rather than death (Park et al., 2001b).

MODEL 3 illustrates my findings to show that low doses of RSV can block hydrogen peroxide mediated signaling.



MODEL 3: A proposed model on the inhibitory activity of RSV on drug-induced death signaling.

Decrease in intracellular O_2^- and cytsolic acidification is a permissive milieu for efficient execution of the apoptotic signal. Pre-treatment of cells with low doses of RSV leads to an increase in intracellular O_2^- that inhibits death signaling by blocking H_2O_2 production and downstream acidification. This slight pro-oxidant cytosolic milieu is inhibitory to effector components of the execution pathway, such as caspases and mitochondrial apoptogenic factors. Alternatively, a slight increase in intracellular O_2^- could directly inhibit caspase activation/activity promoting survival of cells.

6. CONCLUSIONS

1. Vitamin C plays a vital role in the photo-oxidation of MC540 enhancing its photo-degradation under PDT or either pre-activating it with MC540. Photo-products derived from their combination are more stable then those derived from MC540 alone. It is of importance to note that activation of MC540 in the presence of vitamin C {p(MC540+VitC)}showed enhanced cytotoxic potential whereas using MC540 with photo-exposed vitamin C or vitamin C added from outside had little effect on tumor cells. Generation of H_2O_2 was imperative and accounted for the enhanced cytotoxicity of the newly derived compound {p(MC540+VitC)}. Furthermore, {p(MC540+VitC)} photo-product C2 was more stable and better on yield when derived from pMC540 alone.
2. Derived photo-product(s) C1 (merodantoin) or C2 (merocil) generate H_2O_2 that engages the mitochondria by translocation of pro-apoptotic molecule Bax. A direct relation between H_2O_2 and Bax has been established to show that Bax is required for hydrogen peroxide induced apoptosis. This translocation was not CD95/caspase dependent. Furthermore, ceramide that has been strongly linked to H_2O_2 signaling did not play a substantial role in translocating Bax and no new protein synthesis was required for Bax recruitment. Indeed, intracellular H_2O_2 production leads to cytosolic acidification that is the driving force for redistribution of Bax from the cytosol to the mitochondria, a critical event in drug induced apoptosis. Thereby, cytosolic acidification serves as a facilitator in forcing Bax to dismantle the mitochondria for the release of apoptogenic factors like cyt C. Mechanism(s) induced by photo-product(s) of MC540 C1

(merodantoin) are H_2O_2 mediated as pre-incubation with catalase resulted in significant block of caspase activity, Bax translocation, cytochrome C release and PARP cleavage- finally resulting in a complete block of apoptosis as shown by the effect of exogenous addition of H_2O_2.

3. The environment of tumor cells can be changed, such that they can become resistant to drug induced apoptosis. In lieu of our hypothesis, that discusses the critical balance of $O_2^{\cdot -}$ and H_2O_2 that decides the fate of the tumor cell I have classically shown that resveratrol, a chemo-preventive agent at low doses can generate a pro-oxidant state leading to inhibition of H_2O_2 induced apoptosis. Classical drugs that act directly or indirectly via H_2O_2 can fail to induce apoptosis in this environment. However, depending upon the source of $O_2^{\cdot -}$ the effectivity of these compounds can be restored and death signals can be reverted to re-activate the apoptotic machinery by liberating H_2O_2.

Impact of these scientific findings:

The concepts presented in this thesis possess basic as well as bio-medical implications. The major hurdle in treatment of tumor cells is drug resistance. The thesis makes an attempt to explore the object of overcoming drug resistance conferred by tumor cells by survival proteins like Bcl-2 and a high intrinsic pro-oxidant state since tumor cells are more active than normal cells. This biological difference between tumor and normal cells in their redox status might hold the key to therapeutic selectivity and better anticancer strategies can be designed to preferentially kill tumor cells with considerable sparing of the normal cells. This study can be extended to test

the “redox hypothesis” in vivo or in other tumor cell types like solid tumors. In summary, the findings demonstrated in this thesis point to the direction that pro-oxidant state in tumor cells could be switched to an apoptosis favoring state whereby tumor cells will now generate H_2O_2 concomitantly bestowing on them an internal environment (acidic milieu) that promotes death proteins like Bax to translocate to the mitochondria, resulting in efficient apoptosis This approach would serve as a double blow to tumor cells. Thus, it becomes essential to invent biologically active molecules like C1 and C2 that can switch the intra-cellular redox status by generating H_2O_2 and simultaneously engage the mitochondrial death circuitry. The future perspective taken form these findings is exploring ROS targets and to investigate the events critical to the generation of H_2O_2. On the other hand one has to be cautious of drugs (low doses of RSV) in combination chemotherapy that can provide tumor cells with a survival advantage and impede H_2O_2 mediated death signals.

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8. PUBLICATIONS

1. **Ahmad Kashif**, Clement MV, Pervaiz S, "Pro-oxidant activity of low doses of resveratrol inhibits hydrogen peroxide apoptosis.", *Ann. of NY Acad. Sci 2003*; 1010:365.

2. **Ahmad Kashif**, Clement MV, Hanif IM, Pervaiz S,
"Resveratrol inhibits drug-induced apoptosis in human leukemia cells by creating an intracellular milieu non-permissive for death execution ",
Cancer Research 2004 (64):1452-1459.

3. **Ahmad Kashif**, Iskandar K, Clement MV, Hirpara J, Pervaiz S
"Hydrogen peroxide mediated cytosolic acidification is *a* signal for mitochondrial translocation of Bax translocation during drug induced apoptosis."
Cancer Research 2004 (64):7867-7878

4**. Ahmad Kashif**, Lei Z., Pervaiz S, Dramatic increase in photo-oxidation of merocyanine-540 by vitamin C to yield ROS producing agent.
(manuscript under preparation)

II. **Conference Papers:**

1. **Ahmad K.A**. and Pervaiz S., Ascorbic acid accentuates photo-oxidation of merocyanine-540 to generate mitochondria specific photo-product(s). "*93rd Anunal Meeting of the American Association of Cancer Research*"' San Fransisco, CA, USA, 6-10 April 2002. In proceedings of AACR 2002, p.592.

2. **Ahmad K**. and Pervaiz S., Hydrogen peroxide: a signal for Bax translocation to the mitochondria, *10th Euro-conference on Apoptosis*, Institute Pasteur, France October 11-13, 2002 (poster presentation)

3. Clement M-V., **Ahmad K** and Pervaiz S., Low concentrations of resveratrol inhibit H_2O_2 induced apoptosis by creating a non-permissive intracellular milieu in leukaemia cells., *Apoptosis 2003-From signaling to therapeutic tools*, European Conference Center, Luxembourg, Jan 29-Feb 1 2003.

4. **Kashif A. Ahmad**, MV Clement, IM Hanif, Shazib Pervaiz, "Low concentrations of resveratrol inhibits apoptosis induced by known chemotherapeutic agents", *Annals Acad. Of Medicine, Singapore* , Vol.32 (Suppl) No.5. (2003). pg. 98 (poster presentation).

III. **Presentations:**

1. Ascorbic acid increases photo-degradation of MC540, "Meeting new challenges"*2nd GSSFOM conference*, CRC Auditorium, NUS, Singapore in March 2002 (Runners-up prize)

IV. **Awards:**

1. "**Young Scientist Award**" at the **7th NUH-NUS scientific meeting**, October 2nd-3rd 2003 for my work on *"Hydrogen peroxide is a signal for Bax translocation to the mitochondria during drug induced apoptosis*."

9. APPENDIX

1. **Ahmad Kashif**, Clement MV, Hanif IM, Pervaiz S,

"Resveratrol inhibits drug-induced apoptosis in human leukemia cells by creating an intra-cellular milieu non-permissive for death execution."
Cancer Research 2004 (64):1452-1459.

2. **Ahmad Kashif**, Iskandar K, Clement MV, Hirpara J, Pervaiz S

"Hydrogen peroxide mediated cytosolic acidification is *a* signal for mitochondrial translocation of Bax during drug induced apoptosis."
Cancer Research 2004 (64):7867-7878.

Publications above are appended to the thesis.